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Microbial Diversity - a consequence of the
aquatic environment

by

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This thesis is presented for the degree of Doctor of Philosophy

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SUMMARY

Although prosthecate bacteria have been observed in freshwater environments, questions concerning the function or survival value of these integral cellular extensions are unresolved. However, it is now becoming apparent that this group of microorganisms forms part of a highly specialised indigenous population.

An electron microscope survey of the bacterial population from several oligotrophic water bodies has shown that Hyphomicrobium and Caulobacter species constitute up to 20% of such populations, the percentage being correlated with the nutrient status of the system. The multiappendaged genera, at best, approach 1% of the total microbial population. 'Enrichment' systems to which no nutrients have been added, select for the latter. Several isolates of multi-appendaged bacteria have been obtained from such systems. Studies on one of these has shown that gross phenotypic variation can be brought about by varying the nutrient status of the culture medium. These studies have shown that the prosthecae can be induced or repressed by environmental stimuli. This phenotypic variation consequently makes the estimation, simply on morphological grounds, of the incidence of multiappendaged bacteria in the environment difficult. It almost certainly leads to gross underestimates of numbers.

Similarly, studies on Hyphomicrobium have demonstrated that this prosthecate bacterium not only exhibits differentiation which is obligate to the cell cycle, but is also capable of environmentally induced differentiation.

These observations also highlight a fundamental split concerning prosthecal function:- (i) where prosthecal formation is obligate and intimately involved in the cell cycle, e.g. Caulobacter and Hyphomicrobium and (ii) where prosthecae are non-obligate and environmentally induced, e.g. the multiappendaged bacteria, Ancalomicrobium.

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Anne C. Lawrence

Microbial diversity - a consequence of the aquatic environment

<u>CONTENTS</u>	<u>Page</u>
Summary	i
Acknowledgements	ii
Contents	iii
Declaration	vii
 Section 1. General introduction - budding and prosthecate bacteria	 1
Section 2. Bacterial survey of oligotrophic waters	25
2.I Introduction	25
2.II Materials and methods	
(1) Freshwater sources sampled	36
(2) Sampling techniques	38
(3) Enrichment and isolation	38
(a) 'static' enrichments	38
(b) 'attachment' enrichments	38
(c) simple chemostat	38
(d) nutrient enrichments	40
(4) Enrichment, isolation and maintenance of budding and prosthecate bacteria	42
(a) <u>Hyphomicrobium</u>	42
(b) <u>Caulobacter</u>	43
(c) <u>Planctomyces</u>	43
(d) Mushroom-shaped bacteria	44
(e) <u>Ancalomicrobium</u> and <u>Prosthecomicrobium</u>	44
(f) <u>Pedomicrobium</u> and <u>Metallogenium</u>	44
Carbon source utilization	45
(5) Microscopy	45
(a) Light microscopy	45
(b) Transmission electron microscopy	46
(1) Negative staining	46
(2) Shadowing	46
(3) Sectioning - ultrastructural studies	47

	<u>Page</u>
(c) Scanning electron microscopy	47
(1) Preparation of samples	47
(2) Specimen viewing	49
(6) Autoradiography	50
(7) DNA extraction, determination of base composition	50
2.III Present Work	52
(1) Microscopical survey of the microbial flora of oligotrophic waters	52
(a) 'Static' enrichment studies	55
(b) The effect of C ₁ compounds on static enrichments	88
(2) Budding, prosthecate bacteria from oligotrophic environments	90
(a) <u>Caulobacter</u> , <u>Asticcacaulis</u> and the fusiform caulobacters	90
(b) Multiappendaged organisms : <u>Ancaomicrobium</u> and <u>Prostheco-</u> <u>microbium</u>	96
(c) <u>Pedomicrobium</u>	115
(d) <u>Metallogenium</u>	124
(e) <u>Planctomyces</u>	133
(f) The mushroom-shaped bacteria	144
Section 3. <u>Hyphomicrobium</u> . A study on a budding prosthecate bacterium	154
3.I Introduction	154
3.II Materials and methods	165
(1) Source of organisms	165
(2) Media	165
(3) Determination of optimal growth conditions	165
(4) Cultivation	166
(5) Growth requirements	166
(6) Assay for (i) nitrate and nitrite	166
(ii) hydroxylamine	168
(7) Assay for carbon	168

	<u>Page</u>
(8) Gas chromatography	168
(9) Carbon source variations	169
(10) Nitrogen source variations	169
(11) Nitrogen fixation	170
(12) Variations in phosphate concentration	170
(13) Effects of heavy metals - manganese and iron	170
(14) Effect of organic amines and inorganic cations	171
(15) Growth measurements	172
(a) Spectrophotometry	172
(b) Protein determinations	172
(c) Cell counts (plates and chamber)	172
(d) Coulter counter	173
(16) Cell synchronisation	174
(17) Slide cultures	174
(a) Anaerobic slide cultures	174
(b) Aerobic slide cultures	175
(18) Spheroplast formation	175
(19) Light microscopy	175
(20) Transmission electron microscopy	175
(21) Sectioning	176
(22) Bacteriophage isolation	176
(23) Continuous culture studies	176
(24) Uptake and incorporation of label	178
(25) DNA preparation, determination of base composition	178
(26) DNA-DNA homology studies, duplex formation	178
(27) Enzyme assays	179
(a) Extract preparation	179
(b) Hydroxypyruvate reductase assay	179

	<u>Page</u>
(28) Polyacrylamide gel electrophoresis	180
(a) Soluble protein fractions	180
(b) Gradient slab gels (exponential)	180
(c) Gel preparation and electrophoresis	181
(d) Gel staining	182
(e) Standards	182
3.III Results and Discussion	183
(1) Growth and physiology	183
(a) Occurrence	183
(b) Growth conditions	183
(c) Carbon and nitrogen sources, and metabolism	184
(d) Phosphate effects	187
(e) Growth requirements	187
(f) Aerobic and anaerobic growth	189
(2) Life cycle and morphology	189
(a) Synchronisation	189
(b) Life cycle and morphology	196
(c) Batch and continuous culture - population dynamics ?	209
Introduction 1. Batch culture	209
2. Continuous culture	210
Results and Discussion 1. Batch culture	211
2. Continuous culture	220
(d) Stalk synthesis	223
(e) Ultrastructure	223
(3) Bacteriophage for <u>Hyphomicrobium</u>	228
(4) Pleomorphism in <u>Hyphomicrobium</u>	229
(a) Carbon variations and 'lobed' cells	229
(b) Nitrogen source variations and cellular expression	244
(c) The effects of metals	251
(d) <u>Pedomicrobium</u> v. <u>Hyphomicrobium</u>	261
Section 4. Conclusions and future prospects	271
Literature	274

DECLARATION

I hereby declare that this thesis has been composed by myself ,
and has not been used in any previous application for a degree.
The work of which it is a record has been carried out by myself,
unless stated, and all sources of information have been
specifically acknowledged by means of references.



Anne C. Lawrence

Section 1.

General Introduction

Budding and Prosthecae Bacteria

For many years bacteria were classified according to their morphological characteristics, and placed in the categories of cocci, rods, spirals and filaments (Stanier and van Niel, 1962; Skerman, 1967). There were, however, bacteria which were considered to be exceptions, with bizarre and complex cell forms, and often possessing cellular extensions or prosthecae. Prosthecae are defined as semi-rigid appendages extending from a prokaryotic cell with a diameter which is always smaller than that of the mature cell, and which is bound by the cell wall (Staley, 1968). These bacteria possessed diverse physiological characteristics and were collectively grouped together as the 'budding prosthecae bacteria', although some representatives do not fulfil this description (see p. 20). As a group of unusual bacteria, their morphology and life cycles have been reviewed (Starr and Skerman, 1965; Schmidt, 1971; Hirsch, 1974), in an attempt to understand their role in the natural environment.

Electron microscopic surveys of the bacterial population of soil suspensions (Nikitin, 1973; Orenski, Bystrický and Maramorash, 1966a, b; Volarovich and Terent'ev, 1970), and freshwater environments (Staley, 1968) have shown that these morphologically unusual bacteria are ubiquitous to these habitats, but constitute a small proportion of the total bacterial population (Staley, 1971). The bacteria are present in an environment which is constantly changing due to nutrient status, seasonal effects, temperature shifts and stratification, and to cope with this, these organisms have developed unusual morphologies and complex life cycles in order to optimise their growth and reproductive capabilities. The morphology and life cycles of these organisms appears to be a direct consequence of the environment, in that various phenotypes appear to be induced

or repressed by environmental factors. Questions concerning the function or survival value of the integral cellular extensions are unresolved, however. It is now becoming apparent that this group of microorganisms form part of a highly specialised indigenous population. Their unusual morphologies and life cycles have also enabled these bacteria to be used as models for the study of morphogenesis and differentiation (Shapiro, 1971; Dow and Whittenbury, 1976). Morphogenesis defines the changes in external morphology and internal structures of cells in the vegetative cell cycle, and differentiation describes the events, initiated by transcriptional and translational changes, which lead to the formation of a new cell type, e.g. a spore or cyst, where the new cell type may be obligate to the cell cycle, as in Caulobacter, or may be induced by environmental conditions, as in Arthrobacter. Such differentiated cells may revert to their original form, e.g. spore germination, or the differentiation may be permanent, e.g. heterocyst of cyanobacteria. Finally development is a composite event involving morphogenesis and differentiation under intercellular influence, as in some myxobacteria and cyanobacteria, where cells may be modified, and function as part of a multicellular complex.

Frequent studies have been made of differentiation in prokaryotes, mainly due to the wealth of biochemical and genetic information on these organisms (Wright, 1967; Shapiro et al., 1971; Donachie et al., 1973). Extrapolation of any of these models to higher organisms is considered with caution, the main value of these studies being a greater understanding of the processes of these organisms themselves (Bonner, 1973). Relatively few bacteria carry out defined morphogenesis and differentiation, other than cell division, during their normal life cycle, however microorganisms that have been studied previously in this context include Arthrobacter, Geodermatophilus, Chlorogloëafritschii, Azotobacter, Bacillus, filamentous cyanobacteria, Actinomycetes, Myxobacteria, Caulobacter and members of the purple non-sulphur bacteria. These models will now be briefly reviewed in order to consider the environmental parameters which induce morphogenesis and differentiation in these organisms.

Arthrobacter possesses a relatively simple differentiation cycle which consists of a sphere-rod transition that is biochemically and morphologically well defined (Fig. 1.1), the coccoid form is expressed and maintained in a glucose and salts medium, whilst a complex medium or glucose-salts medium with an additional carbon source, e.g. succinate, induces a sphere-rod-sphere transition (Ensign and Wolfe, 1964; Krulwich and Ensign, 1969). Krulwich and workers (1967a, b) were able to show a distinct alteration in the chemical composition of the cell wall mucopeptide during these transitions. Continuous culture studies have indicated that the sphere-rod transition results from a change in growth rate rather than from the presence of an environmental trigger compound (Luscombe and Gray, 1971). Ultrastructural studies on this Gram positive organism have shown that the cell wall of rod cells is considerably thinner than that of coccoid cells, and thus it would appear that cell wall composition is affected by growth rates (Stevenson, 1968; Ward and Claus, 1973). As well as the rod and coccoid cells, in media with high carbon to nitrogen ratio, the cells may be further transformed into enlarged oval or lemon-shaped cells called cystites, which despite their name do not appear to possess resistant properties, as do cysts, their abnormal morphology possibly reflects the presence of a large amount of storage material within the cells (Duxbury and Gray, 1977). To date, no genetic system has been developed for Arthrobacter, so consequently no correlation between genetic events and phenotypic response to the environment has been demonstrated.

Geodermatophilus (Luedemann, 1968) also grows in two major forms, a non-motile, irregularly shaped aggregate of coccoid cells (C form) and a motile budding form (R form) (Fig. 1.2). Growth and division in the C form requires the presence of a factor found in tryptose. Absence of this factor induces differentiation to the R form. Readdition of this factor will again induce differentiation from

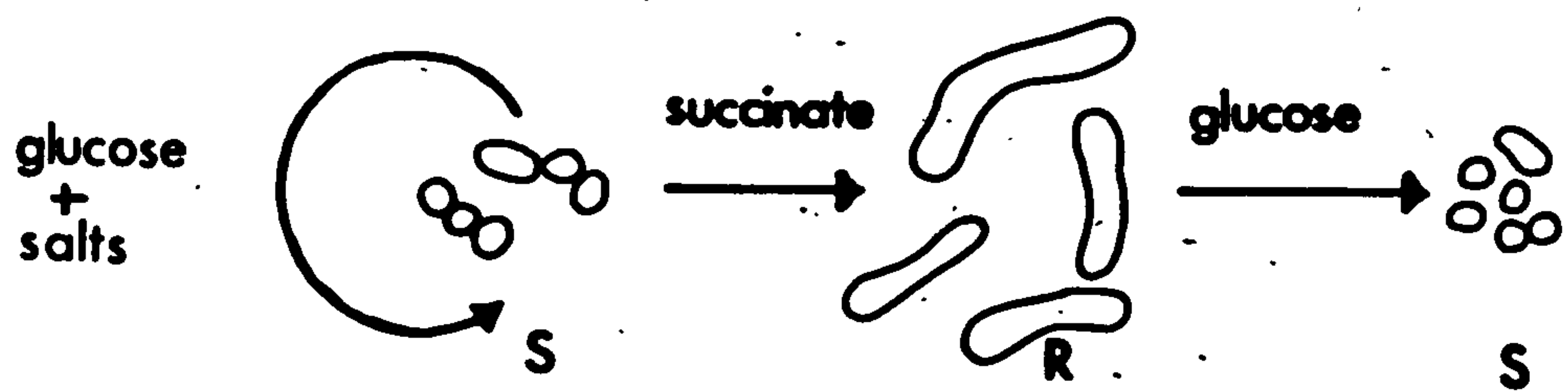


Fig. 1.1 Sphere-rod transition in Arthrobacter

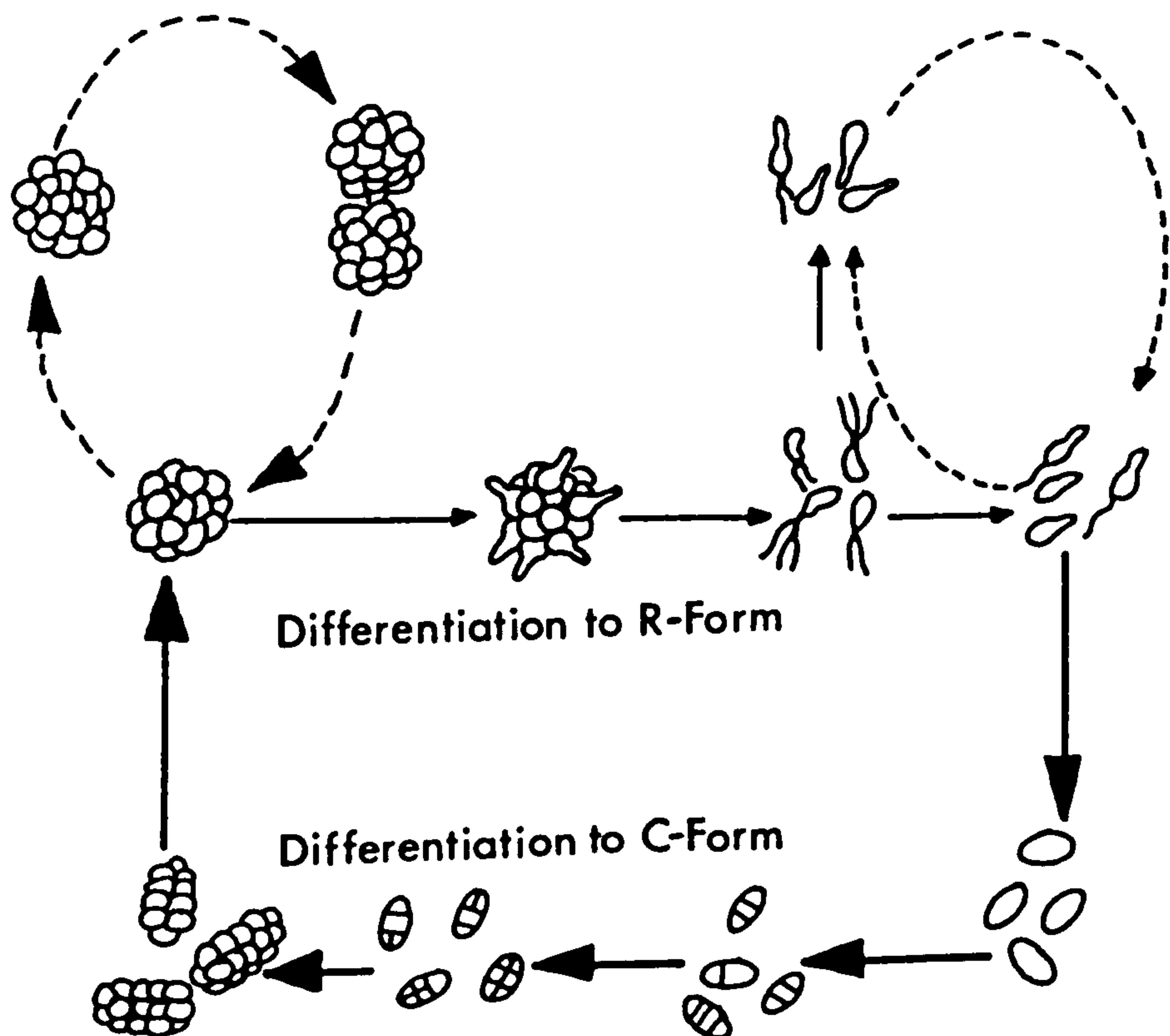


Fig. 1.2 Polymorphic growth cycle of Geodermatophilus strain 22.68 (Ishiguro and Wolfe, 1970).

the R form to the C form. Cellular morphology can be altered by the presence of certain cations and organic amines (Ishiguro and Wolfe, 1970, 1974), which raise the internal pH of the cells, and it has been suggested that this enhances a metabolic event necessary for cell differentiation.

Similarly the blue green alga, Chlorogloea fritschii, exists in more than one morphologically recognisable form, the cellular expression being influenced by environmental conditions (Evans, Foulds and Carr, 1976). Availability of reduced carbon substrate, nitrogen, light and temperature all cause an alteration in the cell type. The two major cell forms are irregular clumps of cells surrounded by a mucilaginous sheath, which are known as aseriates, and filaments (Fig. 1-3). Under photoautotrophic conditions, the filaments predominate (type C) during exponential growth at 34° C. The presence of sucrose (liquid) or complete absence of sucrose imposes aseriate morphology (type B) in both phototrophic and heterotrophic cultures. The removal of nitrate from cultures of C. fritschii leads to development of heterocysts.

Cellular differentiation in Azotobacter vinelandii has also been shown to be under nutritional control (Socolofsky and Wyss, 1962) (Fig. 1.4). The production of metabolically dormant resting cells, cysts, can be induced by using n-butyl alcohol, crotonate or β -hydroxybutyrate as carbon sources (Lui and Sadoff, 1968; Hitchins and Sadoff, 1970). Calcium ions are also believed to stimulate this change in cellular expression (Page and Sadoff, 1975). Because the external inducers of the process are metabolites whose biochemistry is well known, it should be possible to determine the site of initiation of encystment.

The spore forming bacilli have been extensively investigated as differentiation systems which are capable of non-obligate differentiation, i.e. differentiation in response to the environment, forming heat resistant and chemically resistant endospores, which can again become vegetative rods through the process of germination and out-

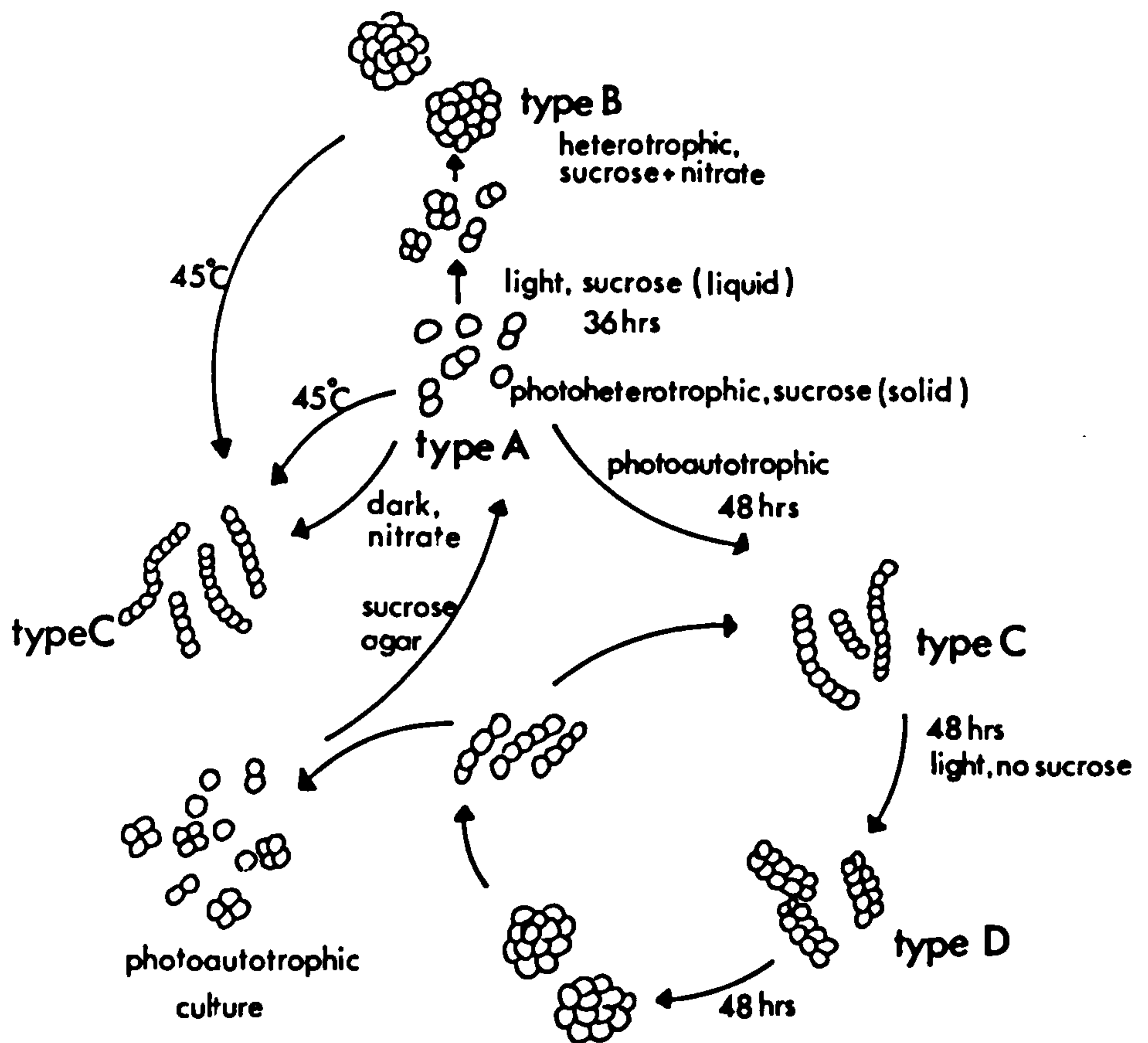


Fig. 1.3 Diagrammatic summary of the morphological variations induced in *Chlorogloea fritschii* by different environmental stimuli.

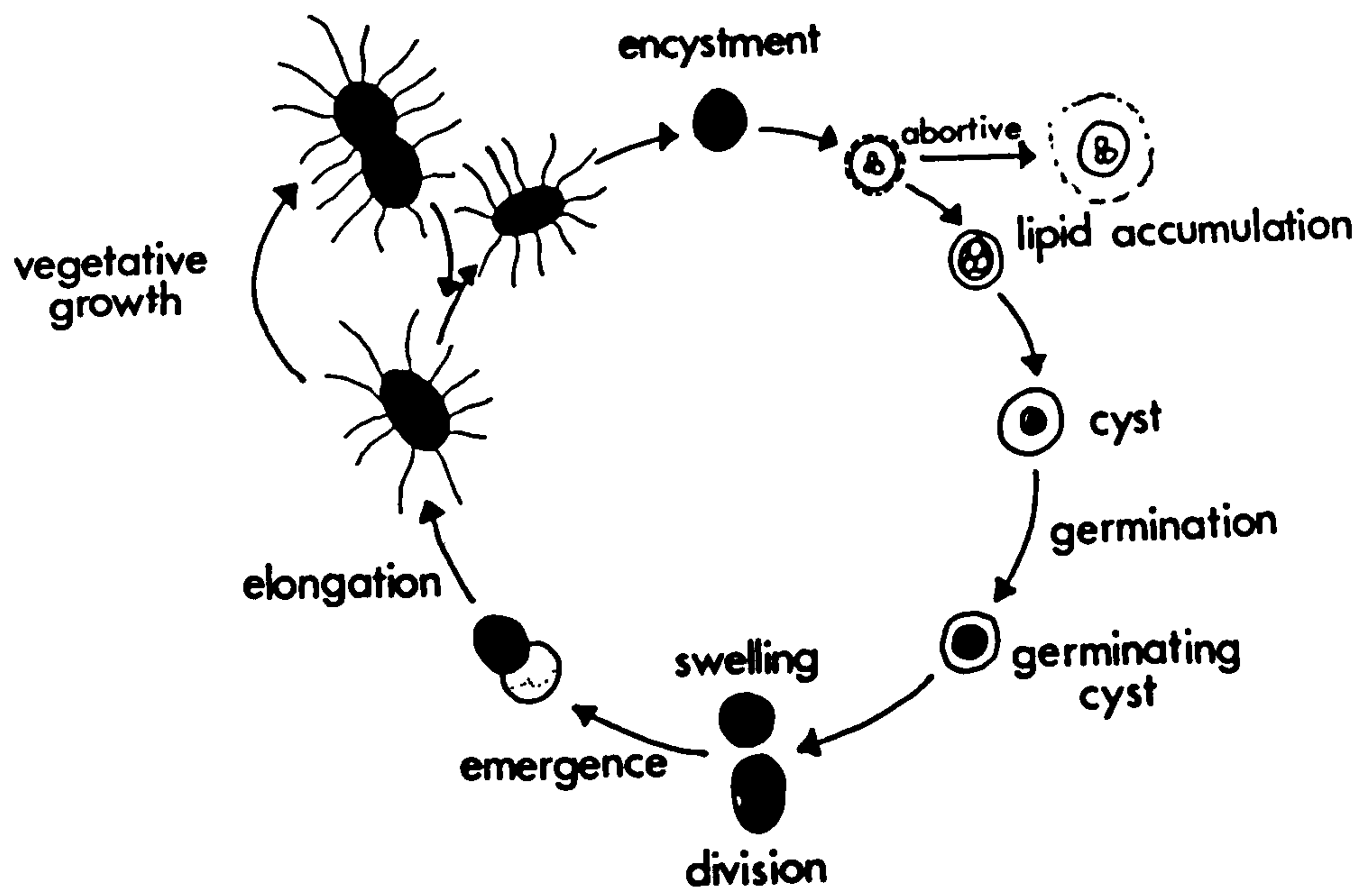


Fig. 1.4 Diagrammatic representation of cellular differentiation in *Azotobacter vinelandii* (Sadof, 1975).

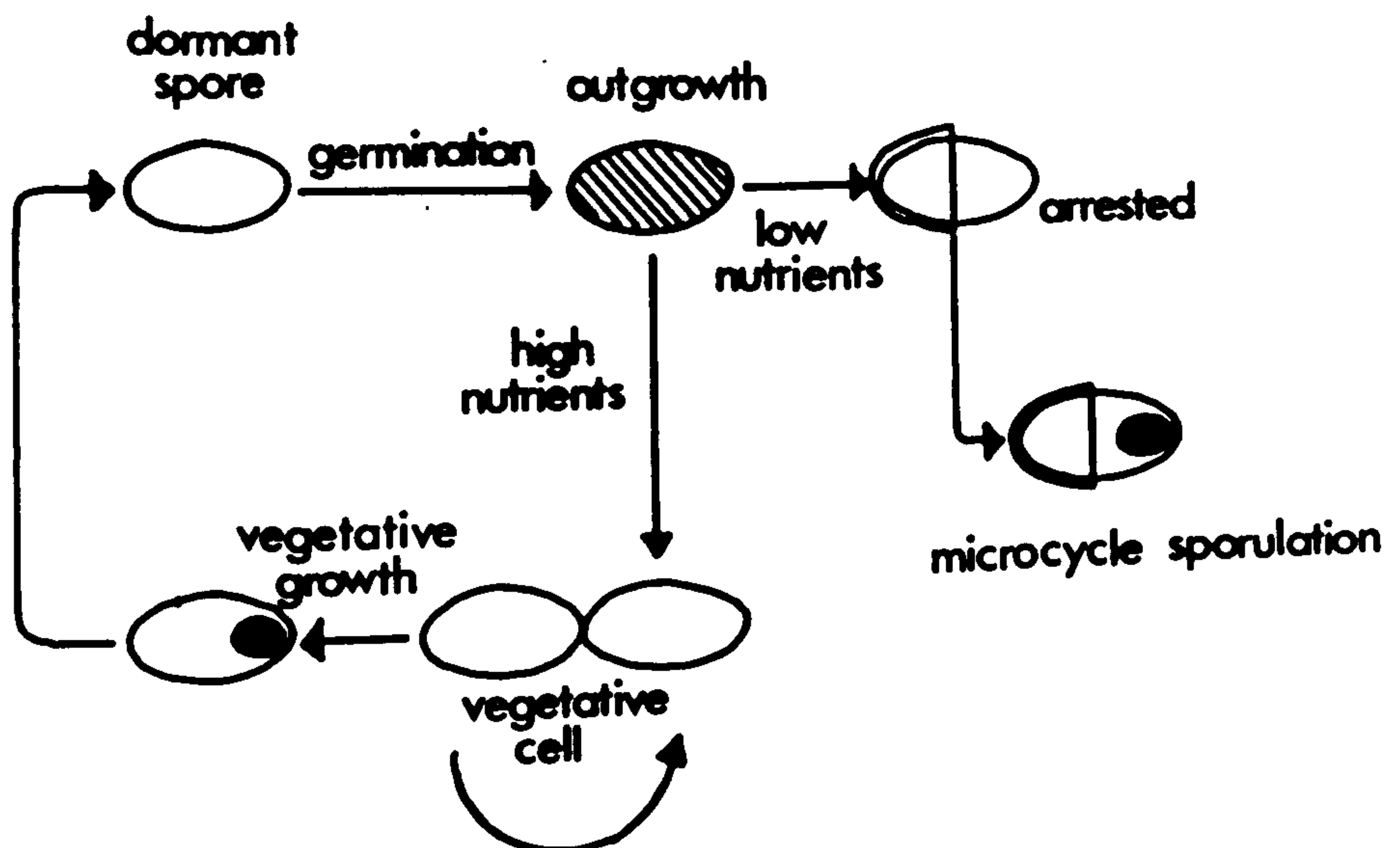


Fig. 1.5 Diagrammatic representation of endospore formation and germination in *Bacillus cereus* (MacKeechle and Hanson, 1968).

growth (Fig. 1.5) (Holmes and Levinson, 1967; Mandelstam, 1969; Szulmajster, 1973; Keynan, 1973). Initiation of sporulation, which can be regarded as consisting of a number of sequential, well-defined chemical and morphological changes, can be effected by starvation for a carbon or nitrogen source (Dawes and Mandelstam, 1970) and sometimes by phosphate starvation (MacKeechie and Hanson, 1968), or by transfer of cells to nutrient deficient medium. It seems likely that initiation of sporulation is in fact a form of catabolite repression (Coote, 1974), where a good supply of carbon and nitrogen is needed for repression, and a deficiency in either allows increased sporulation to occur, the modification being a specific requirement for chromosome replication. Mandelstam, Sterlini and Kay (1971) showed that DNA replication was necessary to effect sporulation. The transcriptional control of sporulation is probably by sequential modification of the vegetative DNA dependent RNA polymerase (Losick, Shorestein and Sonenshein, 1970) together with the sequential induction or derepression of many operons on the genome (Piggot, 1973; Piggot and Coote, 1976). Hutchinson and Hanson (1974) have proposed that a decrease in energy levels in the cells may initiate the derepression of sporulation. Sterlini and Mandelstam (1969) proposed that sporulation is regulated, not only at the transcriptional level but also at the translational level, by demonstrating a sequence of times of commitment to various aspects of the sporulation process, controlled by the synthesis of stable messenger RNA (mRNA), using the inhibitor actinomycin D. Killick and Wright (1974) have pointed out that actinomycin D can, however, inhibit differentiation itself, and hence any interpretation of this result should be carefully considered. However, other studies have suggested the existence of unstable mRNA for sporulation events (Szulmajster, 1973). Thus, at present, there is no clear evidence for any particular controlling factor in sporulation, although studies using mutants have clarified, to a certain extent, the way in which the initiation and subsequent events of sporulation are controlled (Piggot and Coote, 1976).

The filamentous cyanobacteria possess a variety of differentiated structures and a complexity of life cycle unmatched by other bacteria (Fogg, 1949). The two major differentiated cell types that are found in cyanobacteria are the akinete (resting cell) and the heterocyst (Fig. 1.6). Heterocysts are strong candidates for the site of nitrogen fixation and are spaced regularly along the length of the trichome (Carr and Bradley, 1973), the pattern being maintained by a substance produced by the heterocysts, which diffuses along the filaments and inhibits the formation of other heterocysts. It is assumed that the 'inhibitor' is destroyed by vegetative cells, adjacent to the heterocyst (thereby preventing inhibition of its own development) enabling a gradient to be set up about the heterocyst, with new heterocysts developing below a certain threshold level, which is not inhibitory. Ammonia, or a derivative, has been implicated in the control of heterocyst pattern formation. Wilcox, Mitchison and Smith (1973a, b) demonstrated that developing heterocysts (proheterocysts) regress when isolated, presumably as a result of self-inhibition, whereas mature heterocysts do not dedifferentiate. If two simultaneously developing proheterocysts, in the trichome, were too close to one another, one must regress, as the adjacent vegetative cells can only successfully remove inhibitory substance being produced by one heterocyst. The presence of heterocysts in the filament is a direct response to an absence of fixed nitrogen in the environment, however Bradley and Carr (1976) propose that rather than any product of heterocyst function in nitrogen fixation being responsible for the pattern of heterocysts along the filament, an early product of differentiation of a vegetative cell to form a heterocyst may prevent the development of adjacent heterocysts. Heterocysts do not possess the photosynthetic system II (Codd and Stewart, 1977), and so consequently they have become highly specialised cells, dependent on vegetative cells for cellular carbon (Bradley and Carr, 1976). Akinetes have not been extensively studied (Carr and Bradley, 1973), but have been

shown to occur in older cultures, always adjacent to heterocysts. Akinetes possess high levels of phosphate, and alanylglycine and alanylalanine are also present, together with inclusions of sodium acetate. Akinetes contain large numbers of cyanophycin granules, composed of polypeptides of aspartic acid and arginine, as a source of nitrogen. The need of akinetes to accumulate large stocks of cyanophycin could be the reason for their close proximity to heterocysts, the sites of nitrogen fixation in the filament (Simon, 1976). Thus, in this organism, one is observing multicellular co-operation between cells modified for specialised roles, and these morphological variations, which are a direct reflection of the environmental conditions, can be utilised for the study of the 'molecular interpretation' of the environment by an organism.

The actinomycetes and myxobacteria are two further multicellular prokaryotic systems which have been studied as models of differentiation. Chater and Hopwood (1973) and Kalakoutskii and Agre (1976) have reviewed the system for differentiation in Actinomyces. In all but the simplest of these bacteria, germinated spores grow to form branched networks of multinucleated hyphae within the substrate to give a complex colonial structure of closely packed branching hyphae. Aerial hyphae bear terminal spores. Although the genetics of this organism is well understood, the major setback to studies on morphogenesis in Actinomyces has been their failure to undergo their full differentiation in liquid medium, making physiological studies very difficult (Kalakoutskii and Agre, 1976).

Myxobacteria, although prokaryotic, are complex organisms which exhibit cellular and colonial morphogenesis, in a way which is not too dissimilar to that of the cellular slime moulds (Parish et al., 1976). When the nutrient environment becomes depleted of certain amino acids, the individual cells start to aggregate together and give rise to fruiting bodies containing differentiated resting cells (myxospores) (Fig. 1.7) (Ramsay and Dworkin, 1970; Dworkin, 1972). Stimulation of fruiting body formation appears to be a chemotactic response to the environment; in Myxococcus xanthus it can also be effected by exo-

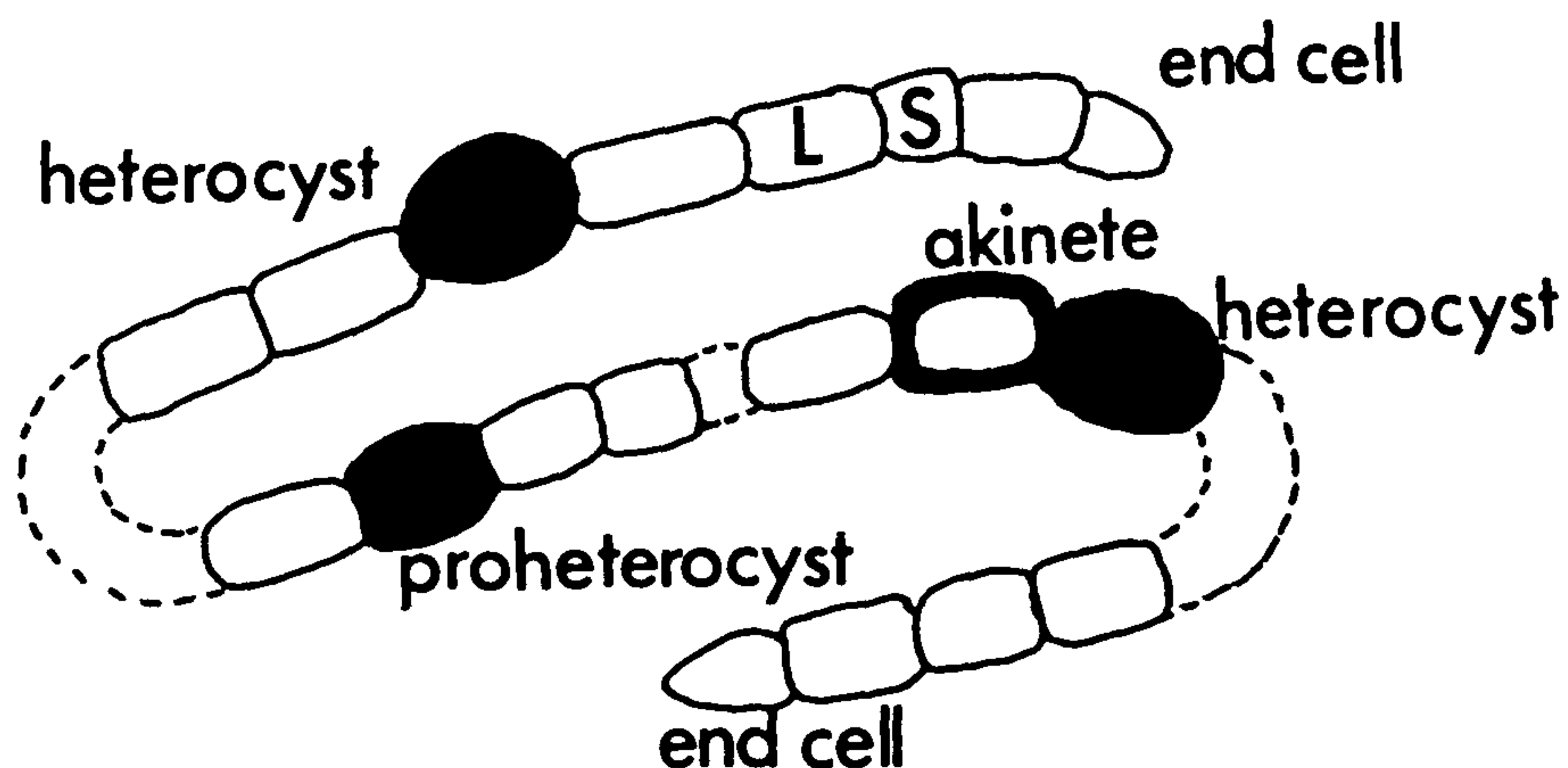


Fig. 1.6 Diagrammatic representation of an *Anabaena cylindrica* filament, showing the constituent cell types. Asymmetric division of the vegetative cells yields a large (L) cell and small (S) cell, which differ in division time (Whittenbury and Dow, 1977).

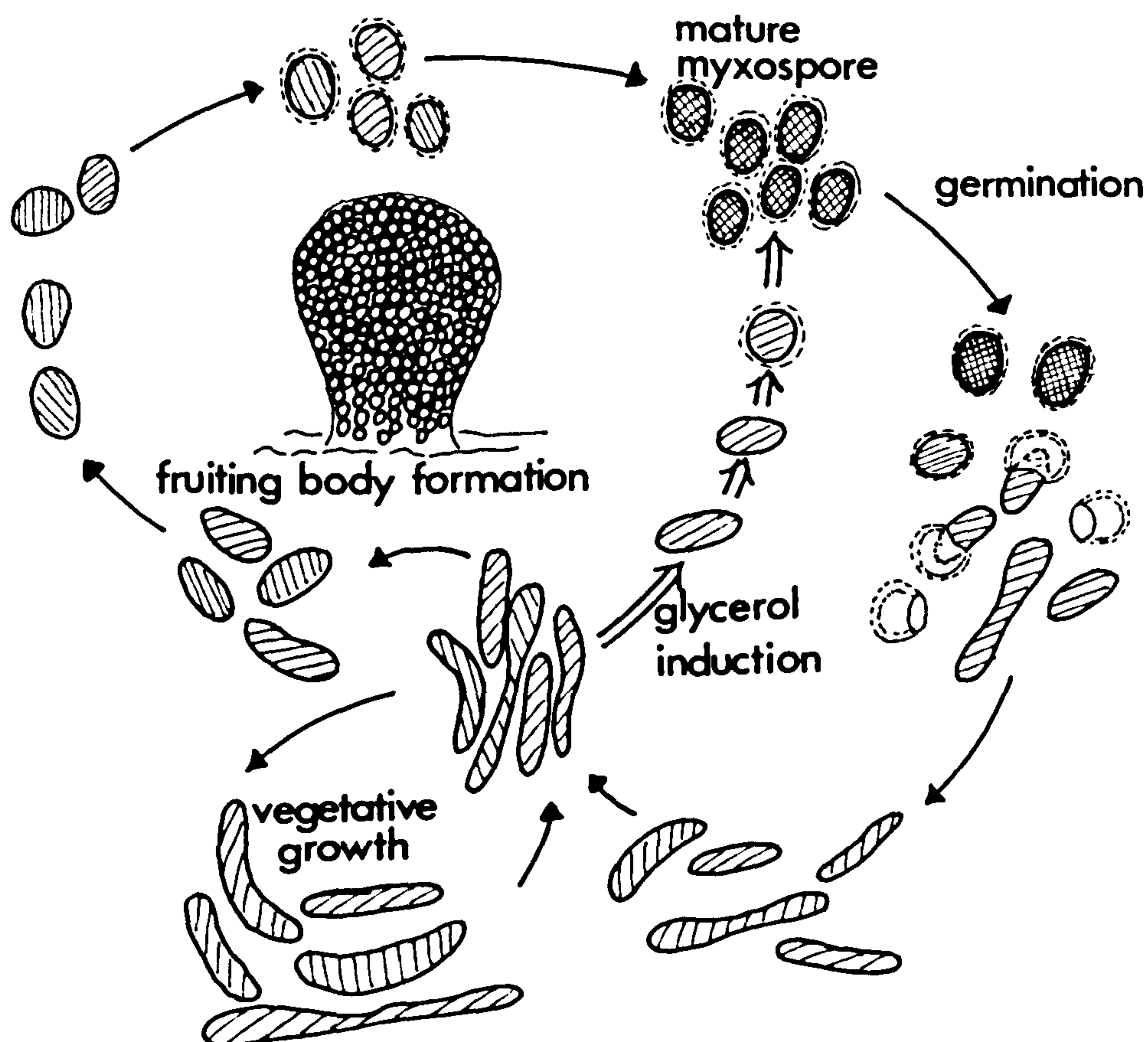


Fig. 1.7 Life cycle of *Myxococcus xanthus* (Dworkin, 1973).

genously supplied cyclic AMP (Campos and Zusman, 1975). The formation of myxospores can be induced in liquid culture by hydroxylated compounds such as glycerol in the presence of the divalent cations, Mg^{2+} and Ca^{2+} (Fig. 1.7) (Dworkin, 1973). In the vegetative phase the cells move by a gliding motility, either singly or in a co-ordinated manner, giving rise to characteristic swarms of cells. Similarly the phototactic response of these cells, as a whole body, is quite dramatic, with marked migration away from the light source, whereas individual cells do not manifest this behaviour, remaining indifferent to the light. The ability of the myxobacteria to aggregate and form elaborate multicellular fruiting bodies is an example of a prokaryotic cell switching from a unicellular to a multicellular mode of development. It should be noted, however, that throughout all stages of the life cycle, the cells are constantly in communal association. Myxospores of Myxococcus xanthus will only germinate at a high cell density ($> 10^9$ cells/ml) or when 1-10 mM phosphate is present in the medium. This is because approximately 10^{10} cells/ml are required for the formation of a new swarm, and 10^{10} cell/ml excrete phosphate at a concentration of about 1 mM, the function of the myxobacterial cell cycle being to maintain at all times the presence or the potential of an optimal density of swarming cells (Dworkin, 1973). These features of Myxobacteria enable it to be studied as a system for morphogenesis and differentiation, cell-cell interaction and the development of a multicellular complex (Parish, Wedgewood and Herries, 1976).

Escherichia coli is probably the best understood organism in terms of its molecular biology, biochemistry and genetics, however it lacks morphological characteristics which can be correlated with biochemical events within the life cycle, with the exception of cell division. The budding and prosthecate bacteria possess well defined obligate differentiation patterns. In fulfilling the requirements for model systems to study cell development, they can be readily synchronised

(Shapiro, 1971; Newton, 1972; Dow et al., 1976; Westmacott, 1976), and grown on defined media (Poindexter, 1964; Shapiro, 1971; Dow et al., 1976) enabling biochemical events to be correlated with morphological development. Also mutants and a system for exchange of genetic material is available for many of these organisms (Shapiro, 1971; Stanley, 1976). Of the prosthecate bacteria, Caulobacter, Rhodopseudomonas palustris and Rhodomicrobium vannielii have been studied as models for differentiation.

Caulobacter, with its obligate life cycle (Fig. 1.8) has proved to be an excellent system for the study of morphogenesis and differentiation (Shapiro, 1971, 1976; Degnen and Newton, 1972a; Wood and Shapiro, 1975). The timing of gene expression, regulation of gene activity and the spatial organisation of gene products within the Caulobacter cell have been studied (Degnen and Newton, 1972a, b; Newton 1972). Genetic exchange systems for transduction and conjugation, and the differential absorption of phage during development have also been demonstrated (Schmidt, 1966; Shapiro and Agabian-Keshishian, 1970; Jollick, 1972). Stalk synthesis in Caulobacter is obligate to the dimorphic life cycle, and although the stalk does not appear to have a direct reproductive function, it is closely involved in the sequential events of the cell cycle, increasing in length with each successive cycle. The stalk is presumed to be an uptake organelle, since its length is directly affected by the concentration of nutrients and the availability of cyclic GMP (Schmidt and Samuelson, 1972). Although Caulobacter fulfils the basic requirements as a model of cellular differentiation (Shapiro and Agabian-Keshishian, 1970), its cellular appendage is not directly involved in daughter cell formation. Members of the Rhodospirillaceae, namely Rhodopseudomonas palustris and Rhodomicrobium vannielii, both possess cellular extensions which are obligate to the reproductive cycle and have dimorphic and polymorphic life cycles respectively, and consequently they have been studied as differentiation models (Fig. 1.9).

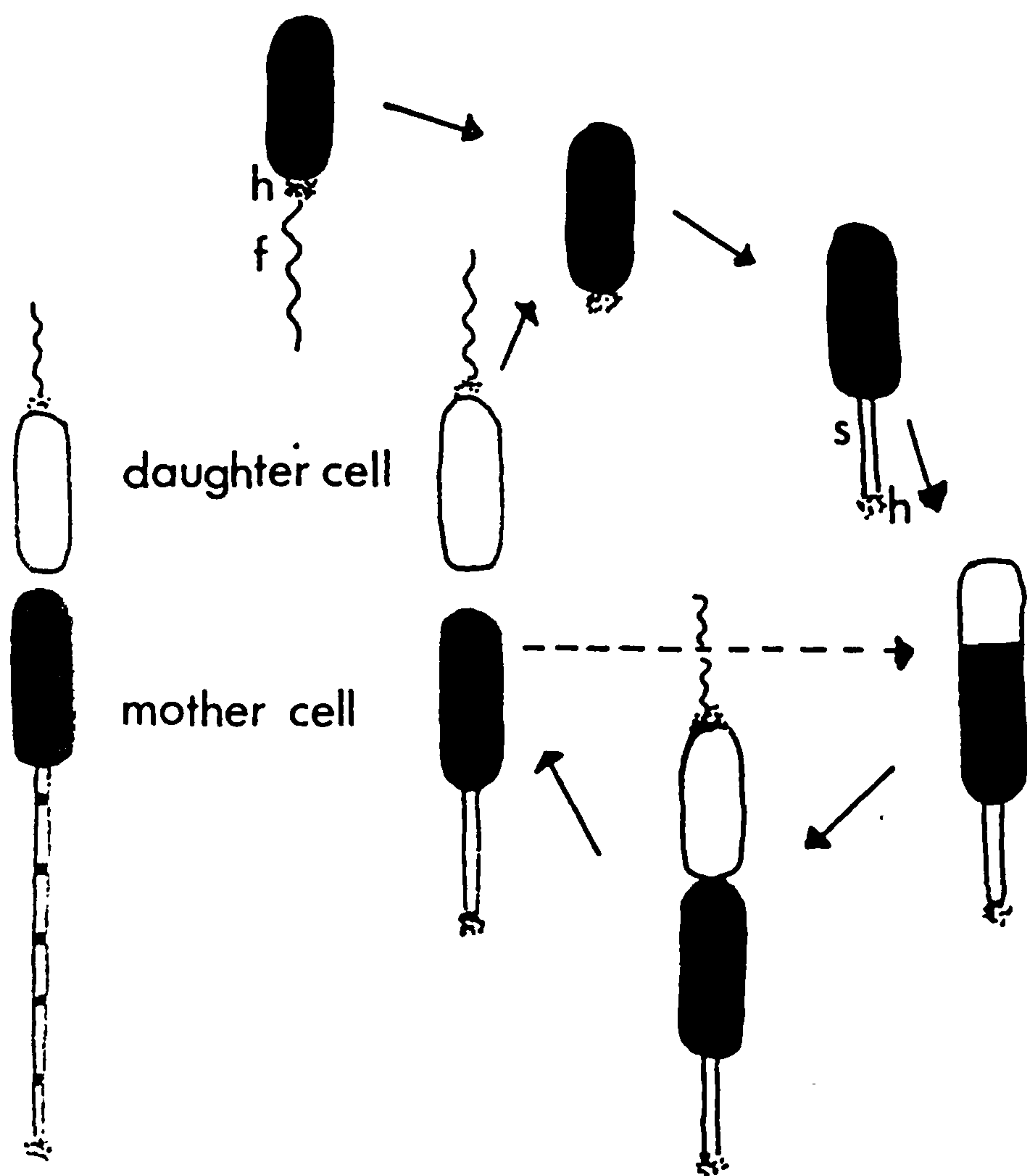


Fig. 1.8 Life cycle of *Caulobacter* sp.

(h = holdfast, s = stalk or prostheca, f = flagellum)

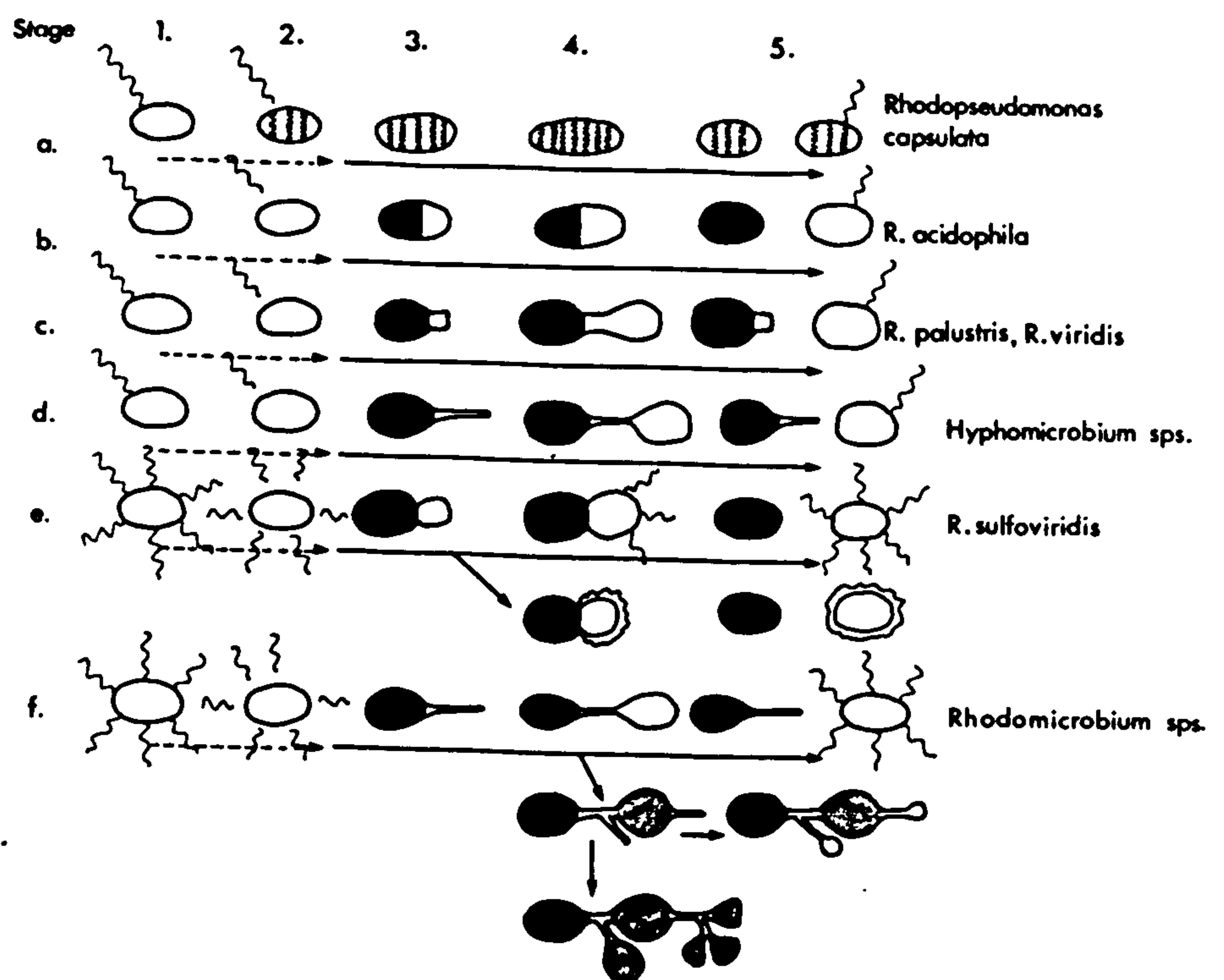


Fig. 1.9 Budding bacteria in ordered degree of morphological and cell cycle complexity. (*R. sulfoviridis*, as described by Gorlenko *et al.*, 1976).

R. palustris has been shown to produce its daughter cell by budding from a tube which develops from one of the poles (Fig. 1.9c) (Whittenbury and McLee, 1967; Westmacott and Primrose, 1976). Tube elongation has been shown to be inversely proportional to phosphate concentrations (Whittenbury and Dow, 1977) in a similar manner to stalk elongation in Caulobacter sp. (Schmidt and Stanier, 1966). Large scale synchronisation of R. palustris is difficult, due to the similarities in morphology between the cell types in the total population (Fig. 1.10).

Rm. vannielli, although physiologically very similar to R. palustris, is more complex with respect to morphology and cell cycle (Fig. 1.9f). Rm. vannielli, grown in/culture, reveals complexes of cells linked by filaments (multicellular arrays) (Fig. 1.11), unattached motile cells (swarmers) and non-motile angular cells (exospores), the expression of each phenotype being dependent upon environmental factors, which appear to trigger the induction of the various cell types (see Fig. 2.2). Its morphology enables large scale synchronisation. Homogeneous swarm cell and exospore populations can be quickly and easily obtained using a glass wool column (Dow, 1974). The temporal expression of molecular events can be correlated with specific cellular morphologies (Whittenbury and Dow, 1977). The mechanism of control at the molecular level of the various cellular morphologies of this organism in response to environmental stimuli has been studied, and it has implicated modification of the DNA dependent RNA polymerase (Dow and Bennett, in preparation). This is a morphologically complex prokaryotic cell which not only exhibits obligate differentiation, but also environmentally induced differentiation (Fig. 1.9f), and as such it is unique. Previously studied models, with the exception of Caulobacter and R. palustris exhibit well defined environmentally induced differentiation, however apart from variability of stalk length and cell volume (Dow and Whittenbury, 1977), environmental pressures do not appear to alter the cell cycle of Caulobacter and R. palustris, other than inhibiting swarm cell development (Shapiro, 1976). To date, only Rm. vannielli exhibits both

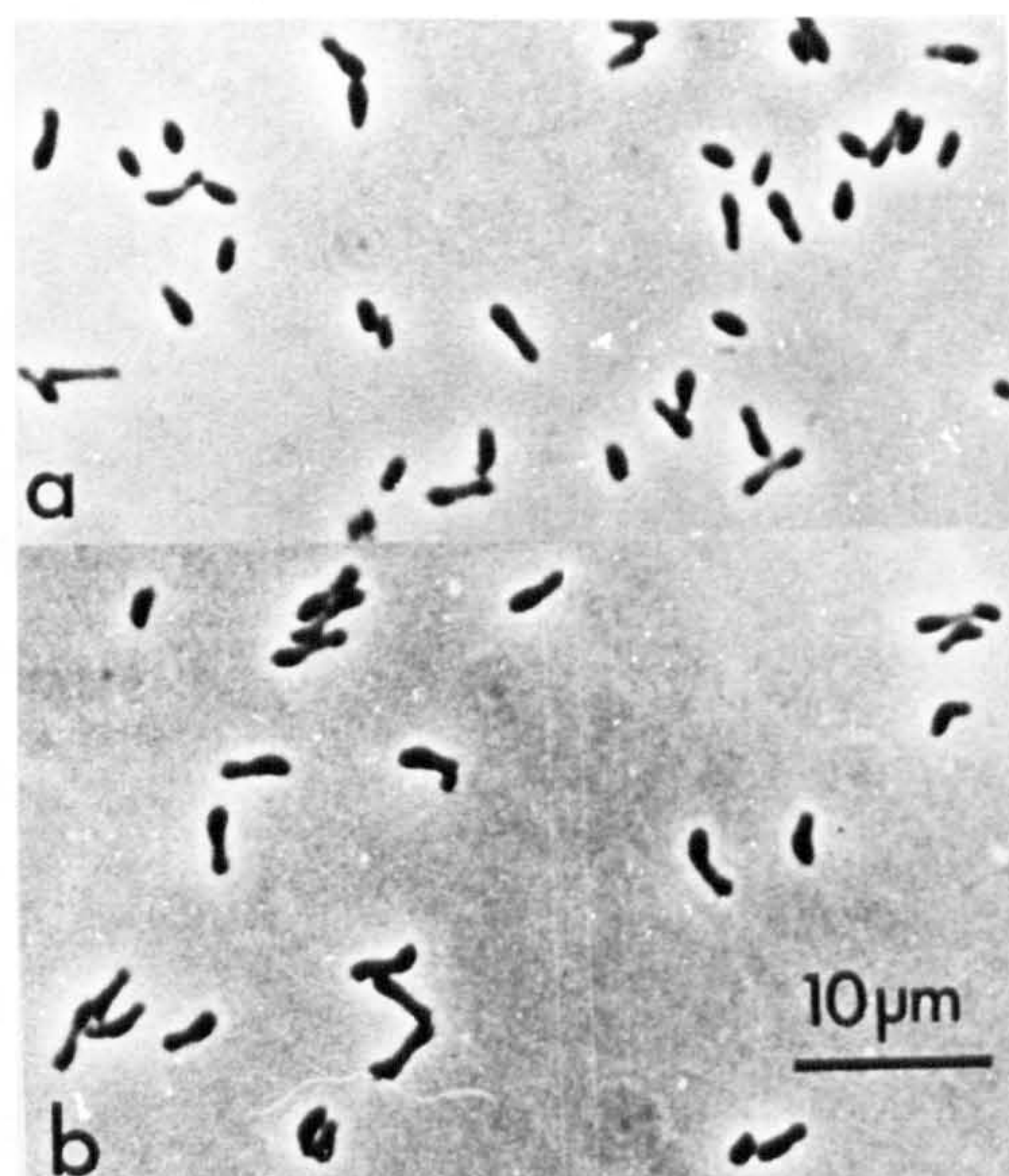


Fig. 1.10 Phase contrast photomicrographs of a heterogeneous population of *R. palustris*

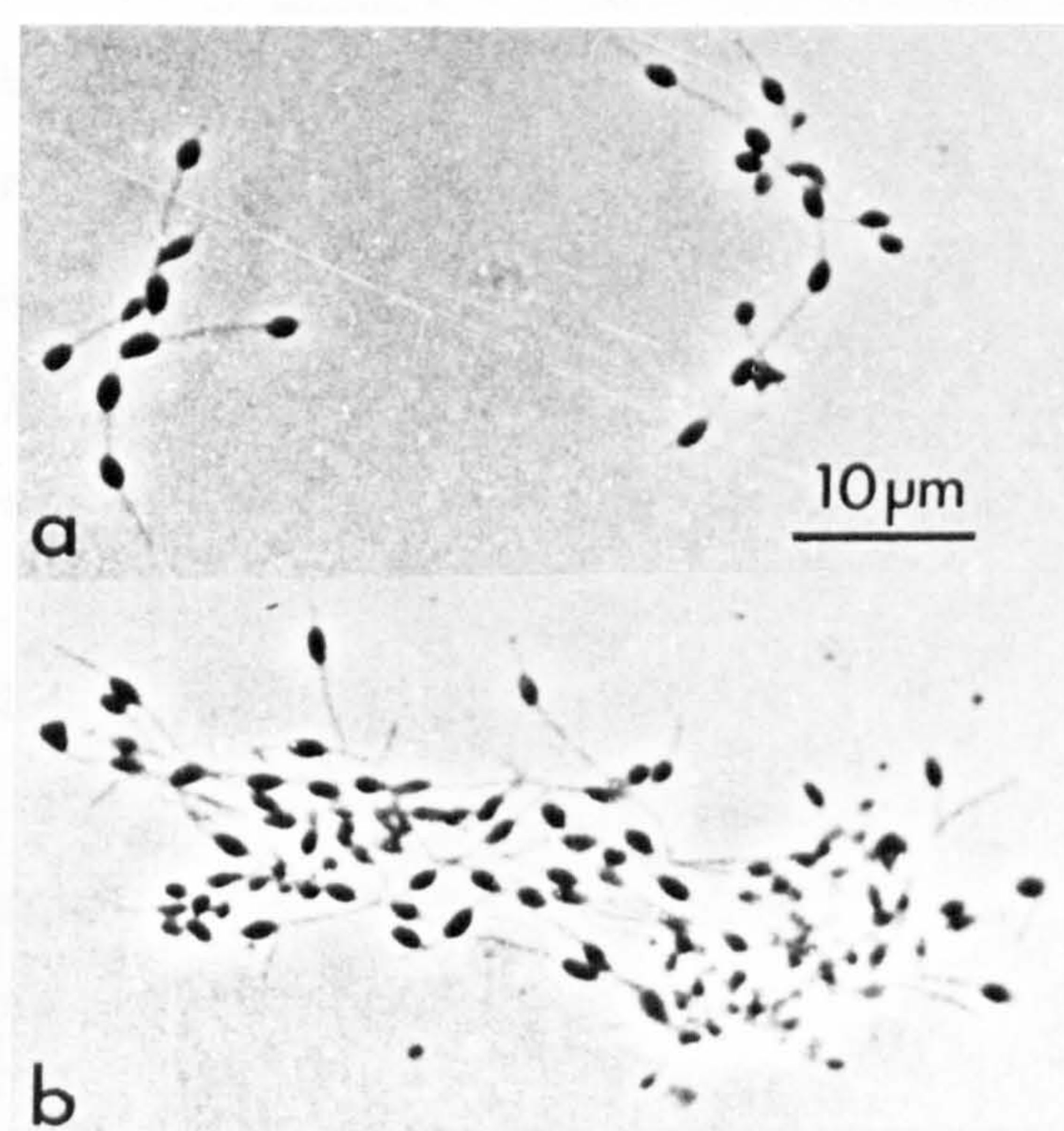


Fig. 1.11 Phase contrast photomicrographs of *Rm. vannielli* in (a) early and (b) late exponential phases of growth.

obligate and environmentally induced differentiation, and as it is clearly amenable to experimentation and analysis, it is proving to be a useful model for exploring the control of cellular morphogenesis and differentiation. As yet, however, no genetic system has been developed for Rm. vanniellii, so correlation of genetic events with phenotypic responses is not possible.

Unlike Escherichia coli, the budding prosthecae bacteria possess more than one distinctive morphological form, this polymorphic cell expression being dependent upon the nutrient status of the environment (Tyler and Marshall, 1967; Dow, 1974; Ishiguro and Wolfe, 1974). As their group name suggests, many of these stalked bacteria reproduce by a budding mechanism, which until recently distinguished them from the rest of the bacterial groups. Whittenbury and Dow (1977) have pointed out that other bacteria can develop by a budding mechanism, that is by asymmetric polar growth, under certain growth conditions (Fig. 1.12). Other workers have shown that for E. coli, when the generation time is in excess of 60 minutes in minimal media, growth is asymmetric from one pole of the cell, i.e. polar; however when the generation time is less than 40 minutes, growth is symmetrical (Fig. 1.12) (Donachie and Begg, 1970; Donachie et al., 1973). The difference between the budding bacteria and E. coli is that polar growth is obligate to the budding bacteria. Also despite the mode of growth, cell separation in E. coli usually results in two identical cells or siblings (Woldringh et al., 1977), whereas with budding bacteria, cell separation generally results in two asymmetric cells, e.g. Hyphomicrobium (Fig. 1.12), although in some instances division is symmetric, e.g. mushroom-shaped bacteria (Whittenbury and Nicoll, 1971). With the exception of chain formation in Rhodomicrobium, where physiological separation of cells is by plug formation, cell separation in budding bacteria is by binary fission.

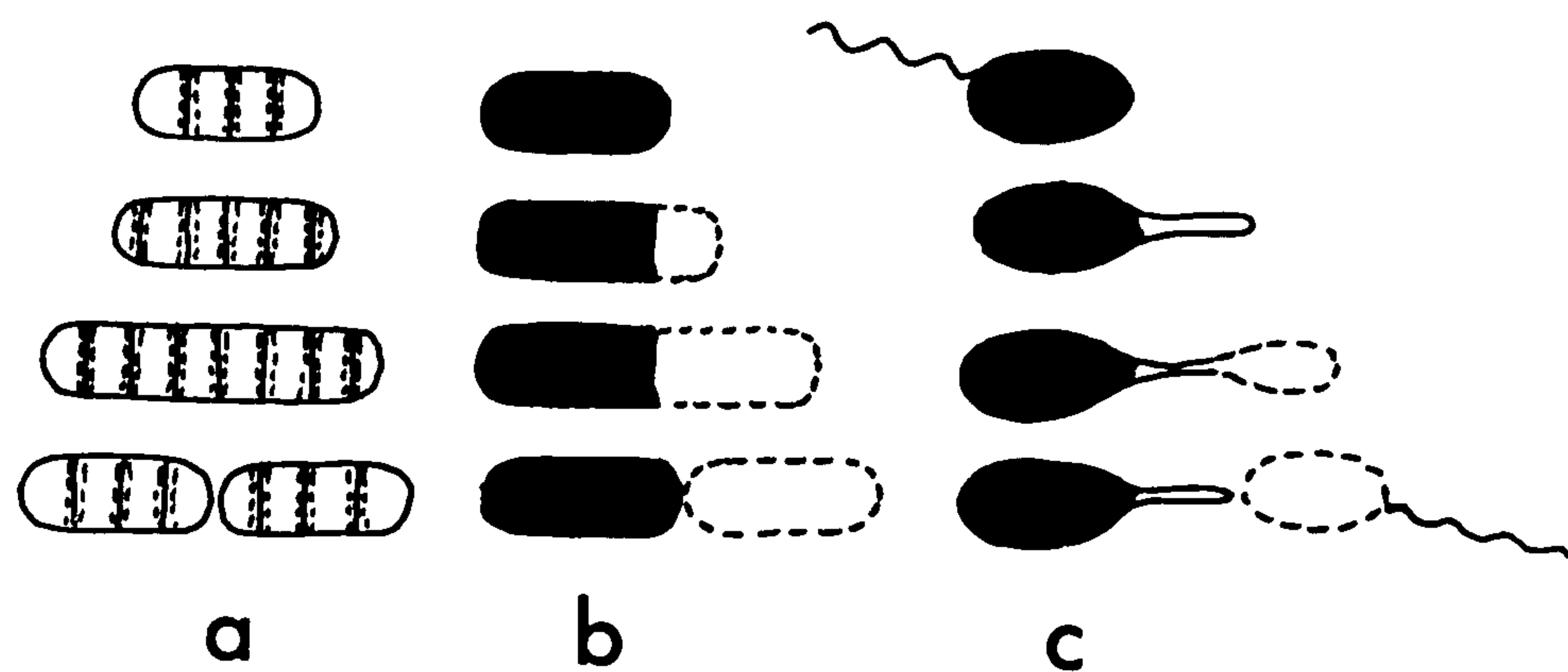


Fig. 1.12 Diagrammatic representation of (a) intercalary growth of *Escherichia coli* with a generation time of less than 40 minutes, (b) polar growth by *E. coli* with a generation time in excess of 60 minutes, (c) obligate polar growth of a budding bacterium, e.g. *Hyphomicrobium* sp. (Whittenbury and Dow, 1977).

Polar growth removes certain constraints as regards shape and internal architecture which govern bacteria like E. coli, allowing these budding bacteria to evolve morphologically. Cells that grow by intercalation are essentially immortal, as old and new material is distributed between the siblings, which consequently appear identical with respect to morphology and internal structures; these cells possess monomorphic life cycles. Polar growth, however, results in the daughter cell being composed of new cell material, whilst material present in the cell when it was formed at the previous division will be confined to the mother cell. Thus the mother cell experiences ageing, which means that the cells are mortal and can undergo only a limited number of reproductive cycles, e.g. Rhodomicrobium 5 (Dow, 1974) only produces four daughter cells from each mother cell. The daughter cells are structurally younger than the mother cells and must undergo obligate maturation (Fig. 1.9) before being capable of reproduction. A polar mode of growth means that the cell is polarised, and develops various cellular structures at predetermined locations on the cell. This can be simple with unidirectional growth, e.g. R. palustris, or complex with bidirectional growth, e.g. Rm. vannielii. Caulobacter, although it does not reproduce by budding, shows obligate polar stalk growth. Consequently polar growth does give these budding bacteria the potential to evolve under environmental constraint, leading to complex polymorphic life cycles in some bacteria, e.g. Rm. vannielii, optimising their growth and reproduction in response to environmental variations.

The budding prosthecate bacteria can be considered as a collective group which includes bacteria which possess prosthecae but in which growth is symmetric, bacteria which lack prosthecae but in which growth is by a budding mechanism, and finally bacteria which fulfil the group description in that they are prosthecates and reproduce by a budding mechanism (Staley, 1974).

Pasteuria (Staley, 1973), Planctomyces (Bauld and Staley, 1975), Nitrobacter (Smith and Hoare, 1968), Gemminger formicilis (Gossling and Moore, 1975) and Rhodopseudomonas acidophila (Whittenbury and McLee, 1967) lack a prosthecal structure, but because of their budding mode of reproduction they are placed in this group. Although Planctomyces (Fig. 1.13) has an extra-cellular stalk, it is distinguished from Gallionella (van Irterson, 1958) and Nevskia (Staley, 1974) by its budding mode of reproduction. Mushroom-shaped bacteria are also included in this group, as growth in these bacteria has been shown to be polar (Whittenbury and Nicoll, 1971).

Prosthecate bacteria, which possess integral cellular extensions but their mode of reproduction is by intercalation, include Caulobacter (Poindexter, 1964) (Fig. 1.14), Asticcacaulis (Pate and Ordal, 1965) (Fig. 1.15), Prosthecochloris (Gorlenko, 1970), Prosthecomicrobium (Staley, 1968) and Prosthecobacter (de Bont and Staley, 1970) (Fig. 1.16). The prosthecae of Caulobacter, Asticcacaulis and Prosthecobacter are obligate and appear to be intimately involved in the cell cycle (Shapiro, 1976) whereas in the case of the multiappendaged Prosthecomicrobium and Prosthecochloris, the prosthecae are non-obligate and environmentally induced (Whittenbury and Dow, 1977).

The true budding prosthecate bacteria include Hyphomicrobium (Zavarzin, 1961), Hyphomonas (Pongratz, 1957), Rhodomicrobium vannielli (Duchow and Douglas, 1949), Rhodopseudomonas palustris (Pfennig, 1969), Ancalomicrobium (Staley, 1968), Pedomicrobium (Aristovskya, 1961) and Metallogenium (Zavarzin, 1964). Buds, flagella and prosthecae develop at specific locations on the cell surface, the exact location being characteristic to that organism. Due to their budding mode of reproduction, the options for development for these bacteria are diverse. Rm. vannielli and Pedomicrobium characteristically develop multicellular arrays, as does Hyphomicrobium under certain environmental conditions (Tyler and Marshall, 1967b), the

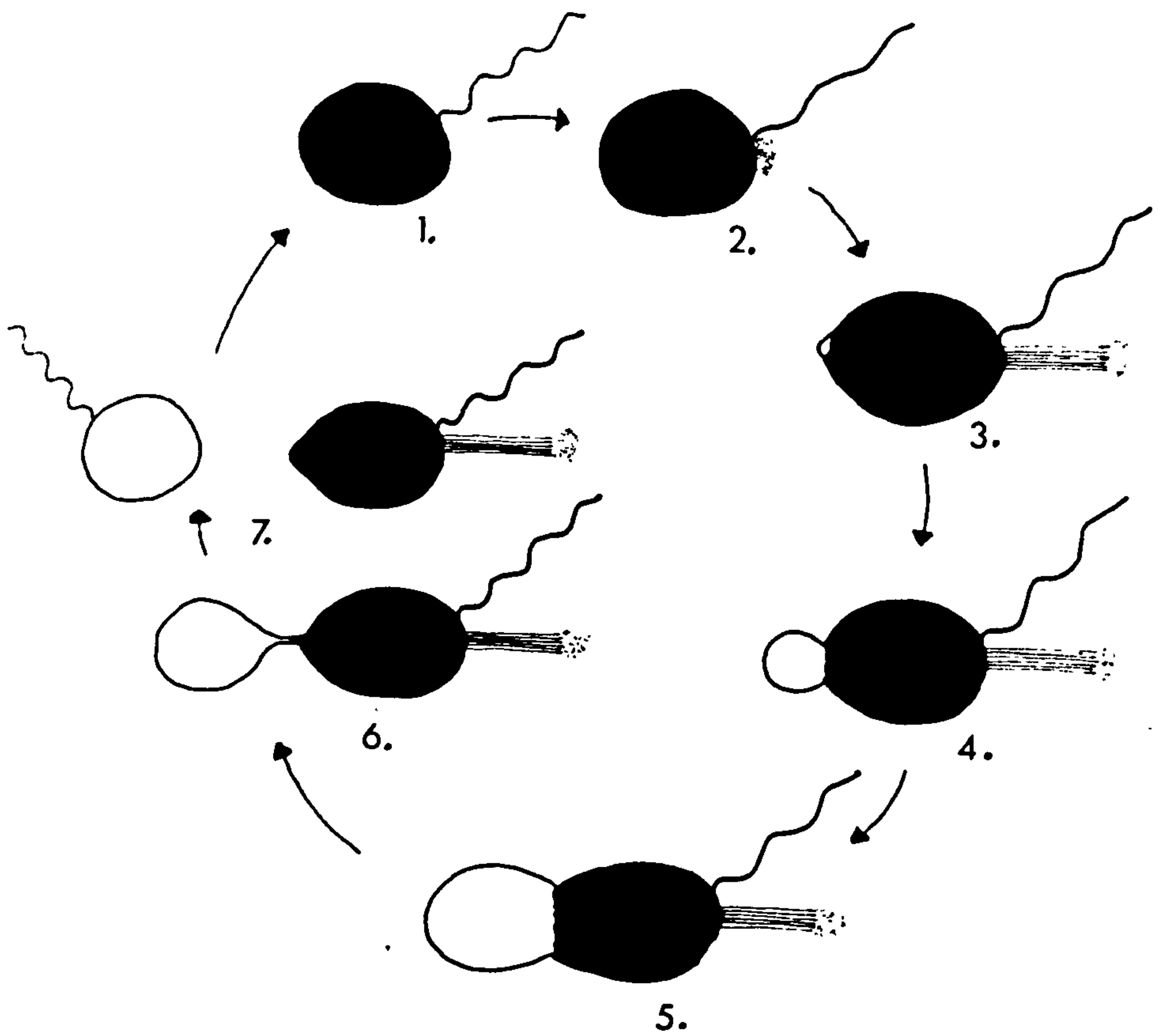


Fig. 1.13 The life cycle of Planctomyces sp.

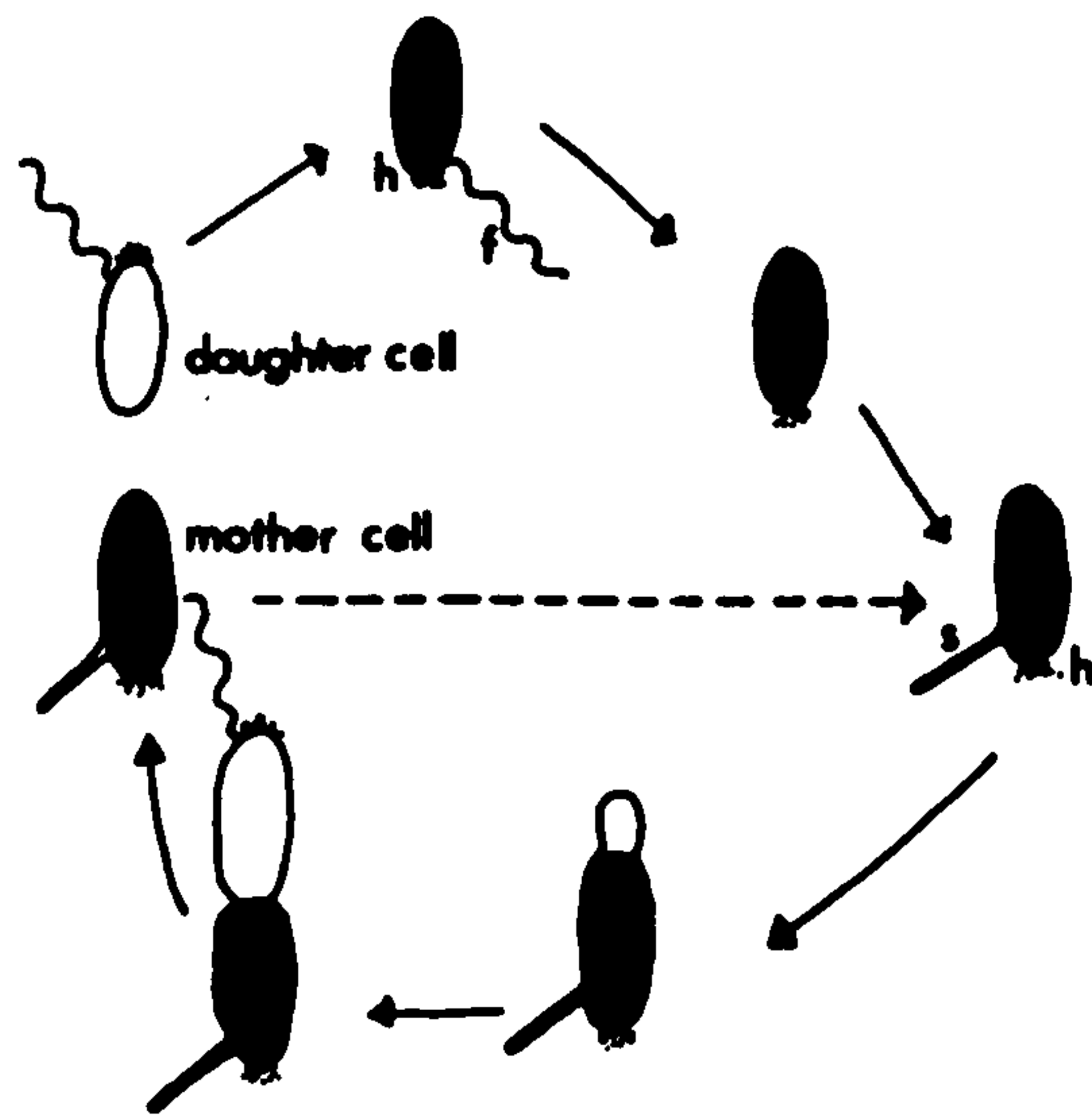


Fig. 1.14 The life cycle of *Asticcacaulis excentricus*

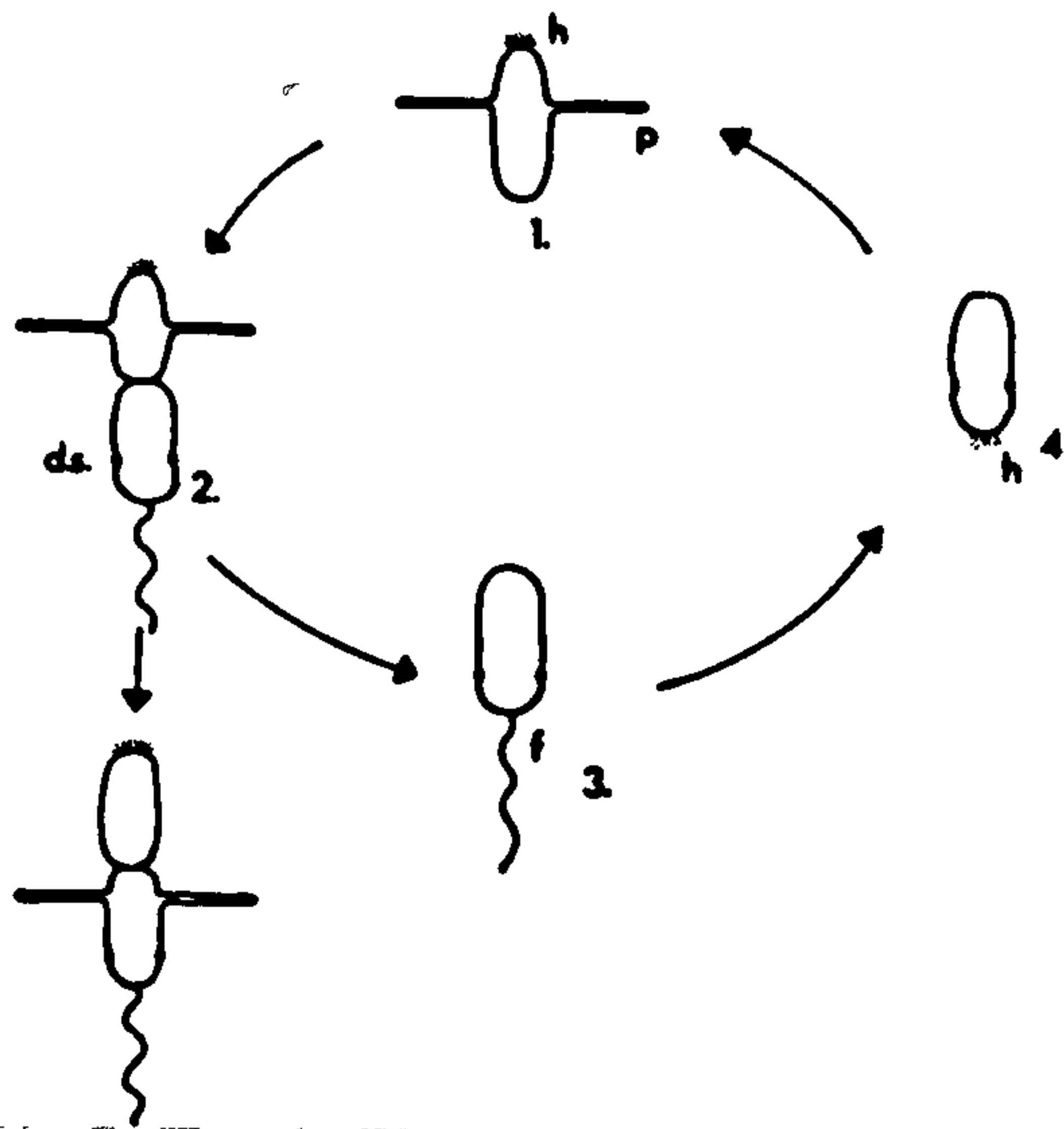


Fig. 1.15 The life cycle of *Asticcacaulis biprosthecum*

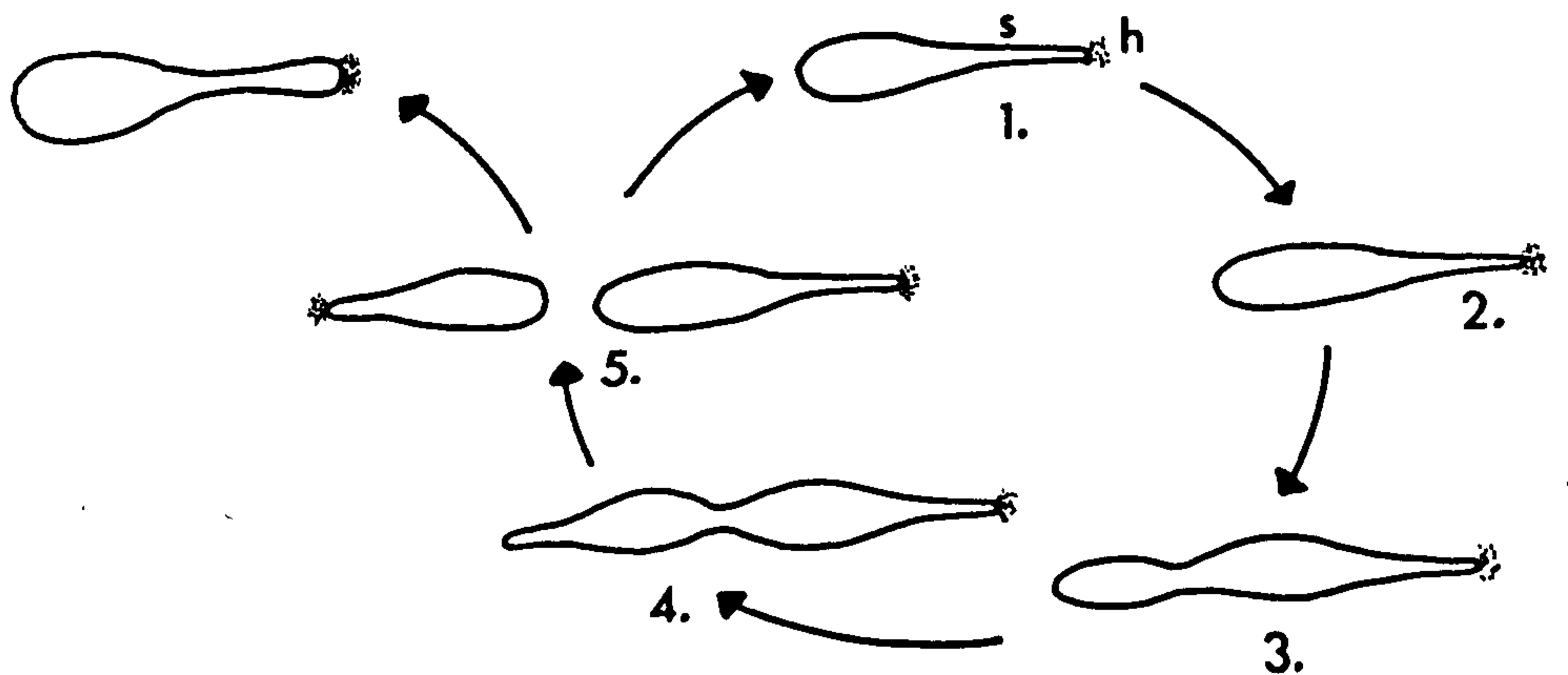


Fig. 1.16 The life cycle of *Prostheco bacter*

(s = stalk, h = holdfast, f = flagellum, p = prostheca)

appendages being intimately involved in reproduction. In contrast, the appendages of Ancalomicrobium are non-obligate and environmentally induced, the buds being formed directly from the mother cell (Dow, 1974).

Studies on Caulobacter, R. palustris and Rm. vannielii have shown that the environment can have a profound effect upon cell cycle expression, inducing morphogenesis and differentiation. By studying some of the other members of this unusual group of bacteria, it is hoped that common principles governing differentiation in these prokaryotic cells may be elucidated. Hyphomicrobium, which has often been referred to as the non-photosynthetic counterpart of Rhodomicrobium (Hirsch, 1964a) was chosen for a detailed study as it has been shown to be capable of a polymorphic life cycle (Bauld, Tyler and Marshall, 1971), and thus exhibits obligate and environmentally induced differentiation. Other prosthecate bacteria, ubiquitous to the aquatic environment, which possess environmentally controlled morphologies, were also studied in order to determine the effect of environmental stimuli on their cell cycles, and to gain some understanding of the role of the budding prosthecate bacteria in the aquatic environment.

Section 2. Bacterial survey of oligotrophic waters

I. Introduction

Previous studies of the microbial flora of oligotrophic waters (Henrici and Johnson, 1935; Zobell and Allen, 1935; Leifson et al., 1964; Hirsch and Conti, 1965; Starr and Skerman, 1965; Ahrens and Moll, 1970; Schmidt, 1971; Staley, 1971; Mallory, Austin and Colwell, 1977) have shown that these bodies of water accommodate a variety of bacteria. It is important to bear in mind that not all these organisms are in their 'natural' environment, as bacteria associated with bottom mud, adjacent land and animal or plant life will also be present (Leifson, 1962). In studies on bacteria which are indigenous to an oligotrophic environment, the presence of prosthecate bacteria has been reported in all cases, although always in low numbers relative to the total bacterial population (Poindexter and Lewis, 1966; Staley, 1971; Mallory et al., 1977).

Unlike typical unicellular bacteria, each genus of prosthecate bacteria contains individuals that are morphologically distinctive and recognisable by phase contrast microscopic examination, although not all of the cell types in the life cycles of the prosthecae may be identifiable, e.g. swarmer cell of Caulobacter, Asticcacaulis and Hyphomicrobium would appear as small rods or vibrios before development of the prostheca. Mistaken identity can also arise due to certain stages in the life cycle of different prosthecate bacteria resembling one another, e.g. stalked Caulobacter appears very similar to some non-budding stages of Hyphomicrobium, which in turn can be mistaken for their photosynthetic counterpart, Rhodomicrobium. Despite these limitations, direct enumeration studies of these morphologically unusual bacteria provide some indication of their numerical significance in their natural habitats (Staley, 1971). Direct electron microscope studies are necessary when considering the budding and prosthecate bacteria as a collective group, as the light microscope imposes certain restrictions when identifying superficially similar microorganisms, e.g. Hyphomicrobium, Planctomyces and Caulobacter.

It is relatively easy to identify a prosthecate bacterium, whose prostheca is an obligate part of the life cycle, however the problems are multiplied when one is considering a bacterium whose appendage expression can be induced or repressed, and this in response to environmental conditions. In this case, simply the method of obtaining the water sample and its conveyance before microscopic examination can affect the nutrient status of the sample sufficiently to influence the morphological expression of these bacteria (Klein et al., 1974; Edwards, 1975).

The morphology of the multiappendaged bacteria appears to be greatly influenced by the environment, and as a consequence they exhibit considerable phenotypic variation. One of these has been studied in detail (Dow, 1974; Dow and Lawrence, in preparation), and appears to be an Ancalomicrobium species, as described by Staley (1968). When these cells were grown on medium containing organic nutrients, at concentrations greater than $175 \mu\text{g/ml}$, there was appendage repression, distinctive pleomorphy, and the cells developed a Y-shaped morphology (Fig. 2.1). This isolate also showed a drastic alteration in prostheca length, number and cell morphology as phosphate levels were altered, and it was possible to totally repress the prostheca formation, ($> 2 \times 10^{-3}$ M phosphate), although the cells were then very pleomorphic and concomitantly non-viable. It is possible that these appendages are uptake 'organelles', capable of being induced or repressed by environmental stimuli, giving this bacterium a distinctive morphology. Studies by Staley (1968) showed that Ancalomicrobium was a multiappendaged bacterium, which reproduced by budding from the cell body, however no mention was made of phenotypic variation and so consequently Ancalomicrobium was only positively identified when it exhibited its classic phenotype. If one were now to consider that these multi-appendaged organisms can be present in the environment in more than one morphological form, and that one form closely resembles

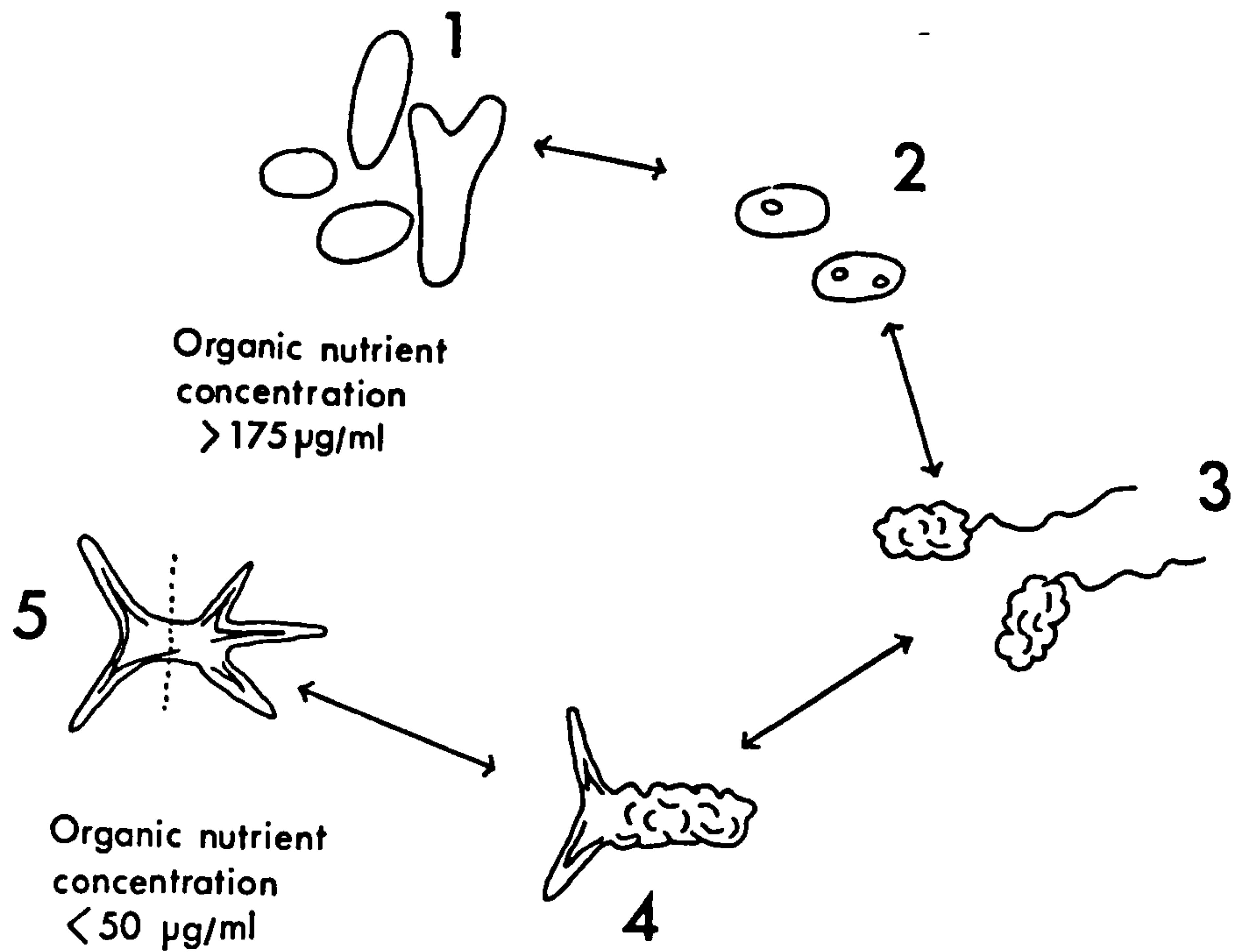


Fig. 2.1 Phenotypic variations induced in a freshwater prosthecate bacterium by variation of the organic nutrient concentration. Each cell type, 1 to 5, was capable of reproducing in a vegetative cycle. The phenotypic variation appeared to be effected by the environment, in this case the organic nutrient concentration.

a rod-shaped cell (type I, Fig. 2.1), it would appear that their numbers could have been grossly underestimated in the past (cf. Staley, 1971).

The multiappendaged bacteria are not alone in expressing phenotypic variation in response to some environmental stimuli, Rhodomicrobium and Hyphomicrobium being capable of polymorphic life cycles under certain environmental conditions. Both these bacteria carry out obligate and environmentally induced morphogenesis within their life cycle. Polymorphism in Rhodomicrobium is well described (Duchow and Douglas, 1949; Gorlenko et al., 1976; Whittenbury and Dow, 1977) and consists of microcolony formation, an alternative 'simplified' vegetative cycle and exospores (Whittenbury and Dow, 1977) (Fig. 2.2). A vegetative culture, expressing chain morphogenesis, will only express the 'simplified' cycle if the light level is low and CO_2 level is high (Fig. 2.2.c). Similarly low light and high CO_2 levels are a requirement for swarmer cell release from the microcolony (Fig. 2.2a). Exospores are produced in a vegetative culture, from the microcolony, when the medium is depleted of an essential nutrient (Fig. 2.2 b). Pleomorphism in Hyphomicrobium has been observed under certain cultural conditions (Hirsch and Conti, 1964; Tyler and Marshall, 1967) with the cells developing into multicellular arrays or giving rise to bizarre shaped cells (see Section 3.III.4).

Assuming identification problems are overcome, one still needs to numerically relate these prosthecate bacteria to the total population. Microscopy, as a method of enumeration, can only give total counts of prosthecate bacteria in the population (Nikitin, 1973; Pagel and Seyfried, 1976), and as the level of debris, including dead cells, can be high, this does not give a true representation of the number of viable bacterial cells present in this environment (Jannasch and Jones, 1959; Floodgate, 1964). Plate numbers give some correlation between viable cells and colonies produced, however they assume that one cell gives rise to one colony and that all the members of a bacterial population

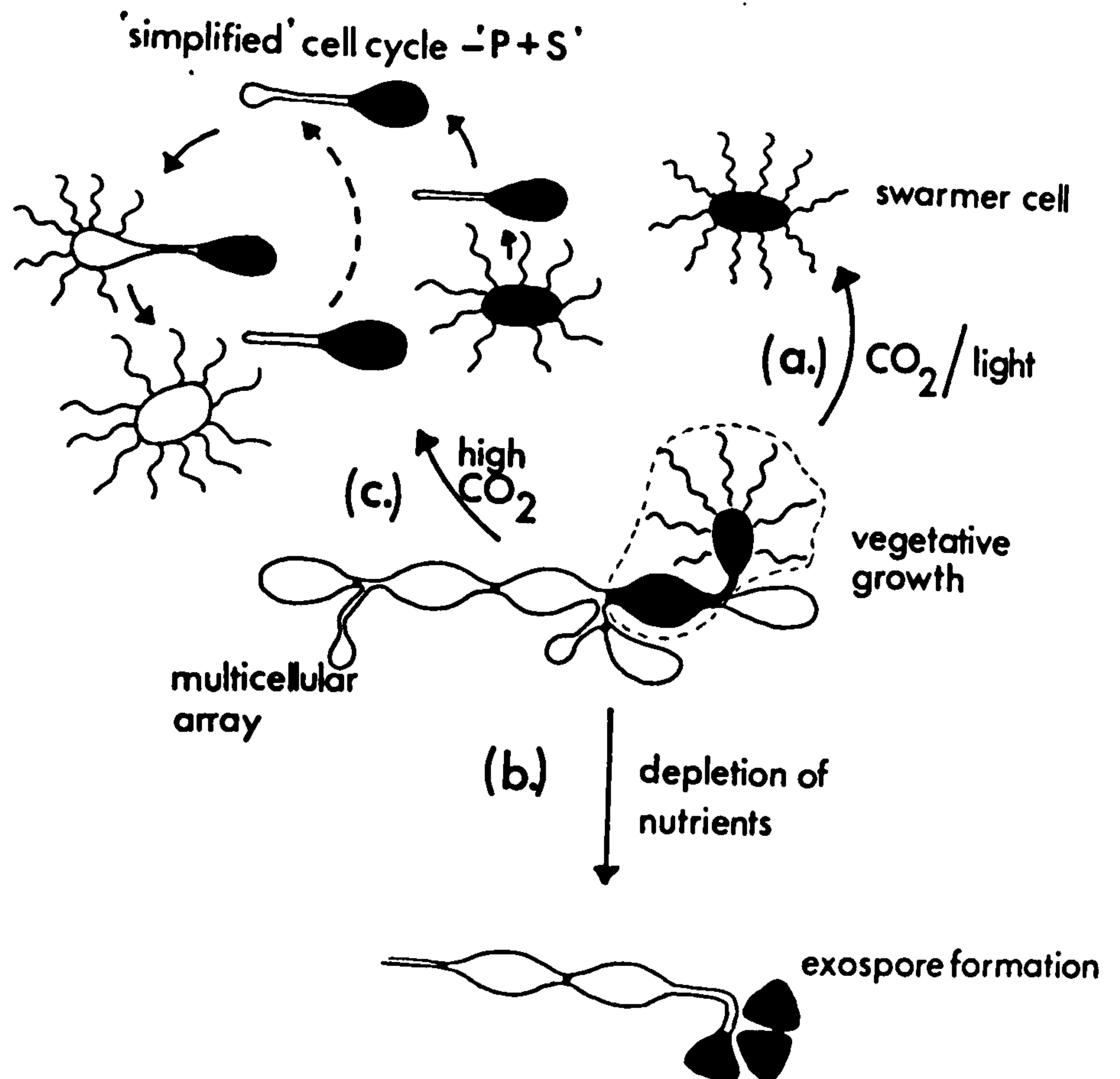


Fig. 2.2 Summary of polymorphism in *Rm. vanniellii*. Vegetative multicellular arrays can develop, (a) releasing motile swarmer cells which can colonise a new area, (b) exospores which possess certain properties of resistance to an unfavourable environment, (c) a 'simplified' cell cycle which resembles *Hyphomicrobium*, depending upon the status of the environment.

will grow on the same solid media (Postgate, 1969). It is well known that different microorganisms differ widely in their cultural and nutritional requirements, and that no one medium can be expected to satisfy more than a small percentage of the microorganisms present in a water sample (Strzelczyk, 1969). Even when solid media is chosen, the effects of pH, oxygen and temperature need to be considered to optimise bacterial recovery from water (Zobell and Conn, 1940; Klein and Wu, 1974). As has been previously mentioned, the phenotype of prosthecate bacteria can be drastically altered, either directly or indirectly by the environment, and so careful consideration needs to be made when using culture medium (Staley, 1968). Furthermore, some prosthecae are unable to grow on rich media, e.g. nutrient broth (Difco), Hyphomicrobium only utilises one carbon and some two carbon compounds, (Attwood and Harder, 1973, 1974, 1978), and consequently the use of such media inhibits their growth and they are not detected. When the prosthecae can grow on rich media, they are usually rapidly overgrown by other members of the population. In general organisms which require prolonged incubation periods or special isolation procedures, in particular conditions of low nutrient concentrations, are frequently overlooked. Aquatic bacteria which grow on media containing significantly low levels of nutrient have not been properly classified or identified (Akagi, Taga and Simudu, 1977; Boylen and Mulks, 1977; Mallory, Austin and Colwell, 1977). Without due consideration to these hitherto neglected groups of bacteria, ecological studies will be limited in providing knowledge of microbial interactions in aquatic systems. To overcome some of these problems, Melchiorri-Santolini et al. (1967) used lake water for culture medium when studying bacteria of a pelagic lacustrine environment. These bacteria were naturally always in a condition of starvation, and although they were growing, their ability to multiply was greatly reduced. By using such poor nutrient medium, these workers were able to culture the bacteria truly indigenous to this particular environment. Staley (1968) has also used filtered and sterilised water from the original sampling area, as a base medium

for the successful isolation of several prosthecate bacteria. Akagi et al. (1977) have isolated oligotrophic bacteria found in the low nutrient environment of the open sea, by the use of a glass fibre filter substitute for agar, the glass fibre containing less impurities compared to the agar used, which can be deleterious to the development of these bacteria.

Before one can successfully culture these morphologically unusual bacteria, they need to be enriched for, preferably in their natural environment, so as not to drastically affect their phenotype. There have been several suggestions that when the organic nutrient concentration is very low, bacteria can grow more readily after having attached to available surfaces (Zobell and Anderson, 1936; Zobell, 1943), presumably because adsorbed nutrients are concentrated and are thus more accessible (Fletcher, 1976). Attachment surfaces have proved useful, as most of these bacteria possess holdfasts or secrete adhesive material, generally known as 'slimes' (Paerl, 1973). Bacterial attachment appears to be by means of a mass of tangled fibres of polysaccharides which extend from the bacterial surface (Fletcher and Floodgate, 1973; Sharon, 1977), or are concentrated at one point to produce a holdfast (Staley, 1974). The adhesion determines particular locations of bacteria in most natural environments (McCowan et al., 1978).

The prosthecate bacteria, as a group, are well known for their holdfast structure (Schmidt, 1971), which enables them to attach to detritus (Paerl, 1973), algae and diatoms (Paerl, 1976), rocks and even pipelines (Tyler and Marshall, 1967). To generate and maintain the adhesive material, generally known as glycocalyx, which may coat the bacterial cell, a bacterial cell must expend energy, and in the protected environment of a pure culture this is a metabolically expensive luxury conferring no selective advantage. Consequently cells which fabricate these sticky coatings or holdfasts are usually eliminated from pure culture by uncoated mutants that can devote more of their energy to proliferation, or the production of glycocalyx is repressed by the

culture conditions (Costerton et al., 1978). Hyphomicrobium, in the natural environment frequently possesses a holdfast on the cell body (see Fig.3.19), however this is often lost in laboratory culturing. In a competitive natural environment, populated by several kinds of bacteria, selection would favour cells, like Hyphomicrobium, that are protected and capable of surface adherence (Tyler and Marshall, 1967; Cagle, 1974; Costerton et al., 1974). Further one must really consider the real functional surface of all cells to be the tangled mass of polysaccharide fibres generated by the cell itself over the total cell surface (cf. Costerton, 1974). It has been shown that a bacterium can anchor itself to inert objects by 'spinning' a mat of polysaccharide fibres that will withstand enormous shear forces. The initial colonization may be accomplished either by bacteria or algae, and in time a complex mixed population of cells builds up within a network of fibres (Costerton et al., 1978).

If the bacteria native to a rushing stream, or water with some degree of movement, were not adherent, the water would be virtually sterile because the bacteria would be swept away much faster than they could swim against the movement of the water. There does, however, appear to be some relationship between this attachment process and the concentration of dissolved organic material, bacteria growing more readily in low concentrations of nutrients, if attached to available surfaces (Fletcher, 1976), presumably because adsorbed nutrients are concentrated and thus more accessible. If one were to consider a square centimetre of submerged surface, as many as a million bacteria might be attached to it, whereas a cubic centimetre of water flowing over the surface might only contain a thousand bacteria (Costerton et al., 1978). The adaptive value of this situation is very clear. The bacteria can extract nutrients from the passing water, and have the waste materials automatically removed by the water, which also provides aeration to the stationary location. Attachment to a surface also protects the bacteria from certain protozoans, and the polysaccharide mat can act as a physical barrier against predatory bacteria and bacteriophage. This clearly

suggests a community structure, with cells of particular species adhering in a favourable niche, close to the source of a necessary nutrient by means of a holdfast structure or more generally by glyco-calyx. Consequently microorganisms which readily inhabit such locations in this manner should, ideally, be studied in such systems, either naturally or artificially created (Geesey et al., 1978).

Slides (Henrici and Johnson, 1935), grids (Houwink, 1952; Hirsch and Pankratz, 1970) and membranes (Swoager and Lindstrom, 1971) have been successfully used, as these minimise the disturbance to the habitat, can remain in the environment under study for indefinite periods of time and direct microscopic examination of the attached bacteria in their natural conformation can subsequently be made. Grids are particularly suitable for this as they can be directly viewed under the electron microscope, copper toxicity from the Formvar coated grids appearing to be negligible (Hirsch and Pankratz, 1970). Attachment surfaces, which have enriched for attaching bacteria, particularly prosthecates, can then be inoculated into filter sterilised water, from the sampling site, or suitable medium, in order to isolate the organisms required (Staley, 1968) (see Section 2.II).

Direct light and scanning electron microscopical observations of freshwater samples have led investigators to conclude that some bacteria may commonly exist in aggregates attached to detrital particles (Paerl, 1973). Water samples may contain numerous aggregates, however large volume water samples overcome the resultant patchiness in distribution of bacteria (Palmer et al., 1976). These aggregates would appear to be 'natural' to this environment, and hence their maintenance in an enrichment system might well prove advantageous to the endogenous population of oligotrophic bacteria.

Continuous cultures have been used to select for microorganisms (Jannasch, 1967), especially those whose morphology can be affected by growth rate, e.g. Arthrobacter's rod-sphere transition (Luscombe and Gray, 1971) and Spirillum sp. (Matin and Veldkamp, 1978).

These studies have shown that the appearance of bacterial cells may be a function of the growth rate, e.g. in Arthrobacter, the cells are coccoid when growing at a low rate, whereas fast growing cells are rod-shaped (Luscombe and Gray, 1971). Organisms growing relatively fast at low concentrations of growth limiting substrates have a relatively high surface to volume ratio (Kuenan et al., 1976; Veldkamp, 1976). In Hyphomicrobium, the stalk elongates with decreasing growth rate, increasing the surface to volume ratio (Harder, pers. comm., 1974). Continuous culture studies have shown that part of the diversity among bacteria in the natural environment is based on selection towards substrate concentration (Wilkinson and Harrison, 1973; Kuenan et al., 1976).

Alternatively two dimensional steady state diffusion gradients have been proposed as a natural method for segregating microorganisms in a mixed population (Caldwell and Hirsch, 1972; Caldwell, Lai and Tiedje, 1973). Exposure of natural aquatic populations to a gradient of pond mud and acetate, within an agar sheet, resulted in the differential growth of a Bacillus sp. and a rod-shaped bacterium with terminal endospores (Caldwell et al., 1973). Because of the changing environmental conditions in these enrichments, the growth of the more tolerant bacteria may lead to the suppression of more fastidious forms. Diffusion gradients thus have an advantage over the continuous culture, used in ecological studies, in that they offer various conditions simultaneously, which otherwise could only be established sequentially. Mixed cultures of the prosthecates, Hyphomicrobium and Rhodomicrobium, together with Thiopedia sp., have been successfully separated (Caldwell and Hirsch, 1972), but as yet this method has not proved successful in isolating prosthecates from a mixed population, from a natural source, possibly because an organism present only in low numbers could be absent from the area in the agar plate that would allow for its optimal growth.

As previously mentioned, enrichments using low levels of added nutrients have been successful in the enrichment and isolation of oligotrophic bacteria, including prosthecates (Staley, 1968), however these do not necessarily select for the bacteria which can survive under "starvation" conditions, as some nutrients have been added, and so consequently these bacteria are readily masked by others whose numbers might be low, but can utilise the added nutrients immediately (Hirsch and Rheinheimer, 1968).

Various systems were considered in this project (Section 2.II), in order to overcome the problem of altering the nutrient and physical status of the system to be studied, when this might have drastic results on the microbial population as a whole (Kahan, 1961). Studies were made on the total microbial population of oligotrophic waters where (a) attempts were made to maintain an environmental balance and (b) the population was 'starved' for a long period (in excess of three years, the duration of the project), to determine how the microbial flora developed as a whole and how the cells adapted to their new environment, under 'starvation' conditions, as described in Section 2.II.

Section 2.II.

Materials and Methods

(1) Freshwater sources sampled

(a) Draycote Water Reservoir. - Situated in Warwickshire, it provides a convenient sampling body. It is reasonably isolated from industrial pollution and the area about it is not excessively agricultural. It lies near to the M 45 motorway, and depending upon prevailing winds it is susceptible to air pollution from motor vehicles. It is an artificial reservoir with a clay basin of maximum depth 70 ft. and covers 600 acres.

(b) St. Paul's, Rugby. - Again situated in Warwickshire, this private body of water consists of three fairly eutrophic ponds leading into a fairly shallow oligotrophic lake. The ponds are fed from a stream, whose source is in nearby woodland.

(c) The Lake District, Cumbria. - Several of the lakes were sampled periodically, (Map, Fig.2.3). Wastwater, Ennerdale and Haweswater were preferred, as these were isolated from agriculture, with only forestry and rough grassland in the vicinity, and constituted oligotrophic water bodies. Estwaite water was sampled as an example of an enriched body of water, surrounded by farmland. Thirlmere was sampled as an intermediate between oligotrophism and eutrophism, being a reservoir with no access to gathering grounds. There are several small tarns (sample sites 5, 6 and 7) situated about 500 ft. above the Natural Environment Research Station at Sawrey, on Lake Windermere, which are surrounded by rough grassland and some forestry, which were sampled, because unlike many of the bodies of water in the Lake District, these were devoid of human as well as agricultural and industrial pollution (with the possible exception of occasional grazing cows and atmospheric pollution).

(d) The Roman Baths, Bath . - Water samples were also collected from the underwater springs which feed the Baths.

(e) Water samples were collected from random water sources in Warwickshire throughout the project, although not on a routine basis.

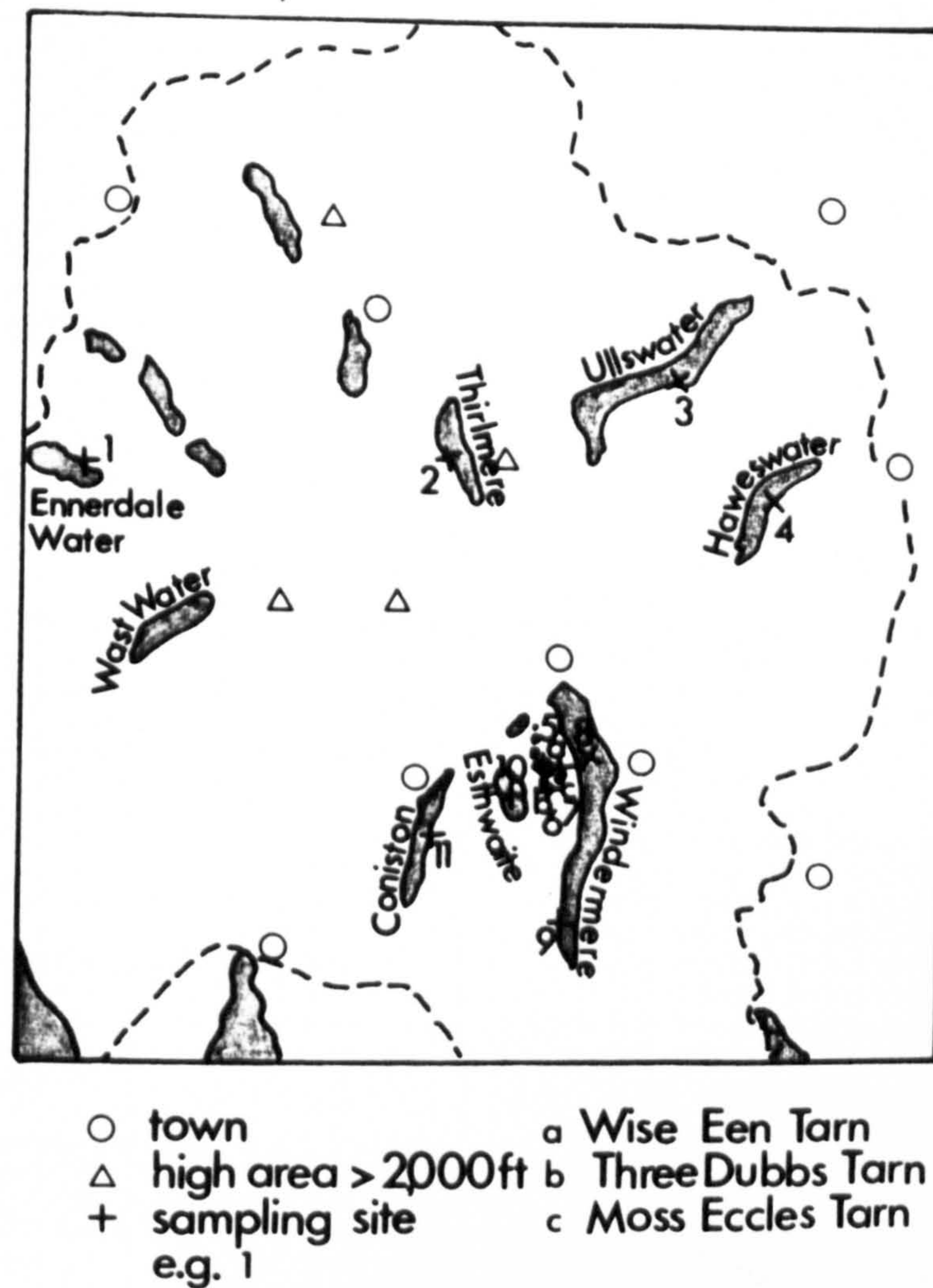


Fig. 2.3 Outline of major lakes in the Lake District, Cumbria, showing sampling sites, relative to towns and relief of the area.

(2) Sampling techniques

Where possible, sampling was done away from the water bank or shore, using a boat, to collect samples from the surface and from various depths, at the same location (Edwards, 1975). Samples were collected using a Casella water sampler (Casella and Co. Ltd.). The copper container holds 250 ml glass bottles, which were sterilised before use. Water samples were taken from the air-water interface and then at 0.5 m intervals, and maintained at 4⁰ C, until they could be studied in the laboratory. (This was usually less than 2 hours).

(3) Enrichment and isolation

(a) "Static enrichments"

"Enrichment" systems to which no nutrients were added, were set up in 2 litre glass flasks. 1 l. of water sample was placed in a sterile 2 l. flask and plugged tightly with cotton wool. This was then left undisturbed at room temperature on a window sill to receive daylight but not direct sunlight, or on a shelf in a room with little available light, for a period of up to 3 years.

(b) "Attachment enrichments"

Suitable attachment surfaces were added to 2 l enrichment flasks. Sterile copper grids (Agar Aids Ltd.) coated with Formvar plastic, and sterile nitrocellulose membrane filters (Shandon Southern Ltd.) were carefully floated on the water surface. Glass coverslips and slides, washed in chromic acid, water and finally ethanol flamed, were suspended into the water in the flasks by means of cotton and metal clips, so that they were just submerged (Henrici and Johnson, 1935).

(c) Simple chemostat

The basic fermenter unit employed a 100 ml side-arm flask with a working volume of 70 ml. There was no provision for a pH or temperature control, all runs were performed at 30⁰ C in a warm room with a magnetic stirrer and follower providing agitation (Fig. 2.4). Sterile filtered air or oxygen free nitrogen was introduced into the apparatus via a syringe needle, depending upon the conditions of incubation. Culture samples were taken from the overflow, using a sampling hood and sterile Universal bottles. Media was added via a bulbous column

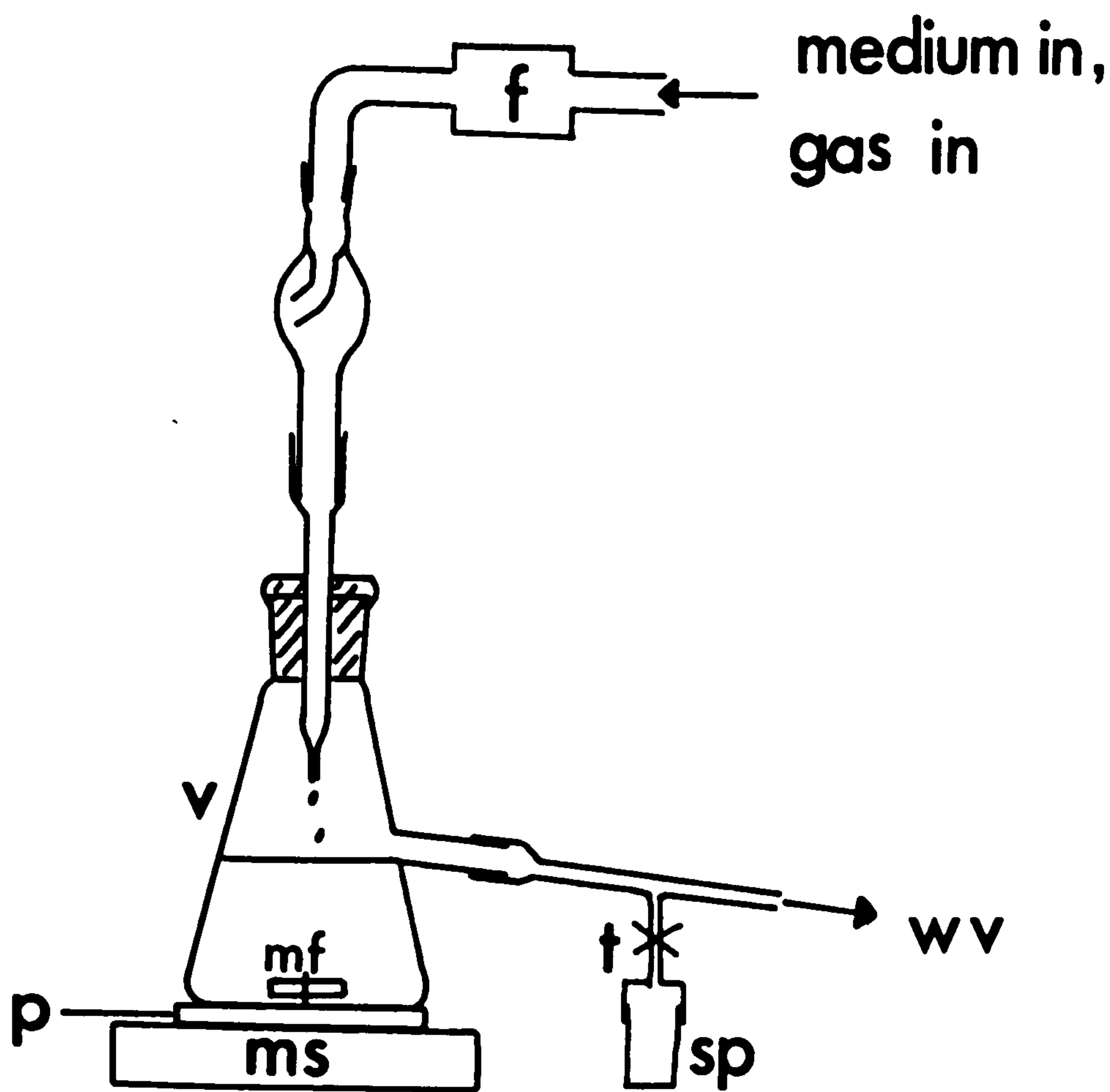


Fig. 2.4 Simple chemostat

Routinely employed in bacterial enrichments, the constant flow of medium, 'mimicking' the natural freshwater situation. Inoculum contained bacteria and algae.

v = vessel

f = filter

p = polystyrene block

ms = magnetic stirrer

t = tap

sp = sample port

wv = waste vessel

mf = magnetic follower

to avoid backgrowth. HB media was routinely used with methanol at a final concentration of 0.001% (v/v).

(d) Nutrient enrichments

Media included the following components, made up as stock solutions:-

Phosphate buffer:

$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	31.2 g in 1 l of distilled water
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	71.7 g in 1 l of distilled water

(Add 500 ml of each salt to 1 l of water to give 0.1 M PO_4^{2-} , pH 6.8-6.9)

Mineral base:

KNO_3	5 g
NH_4Cl	1 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	4 g
NaCl	0.5 g
$\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$	0.5 g
Distilled water	1 l

Trace element solution (Pfennig, 1967) :

EDTA (disodium salt)	500 mg
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	200 mg
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	10 mg
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	3 mg
H_3BO_3	30 mg
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	20 mg
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	1 mg
$\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$	2 mg
$\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$	3 mg
Distilled water	1 L

Vitamin solution (Staley, 1968) :

Biotin	2 mg
Folic acid	2 mg
Pyridoxine HCl	10 mg
Riboflavin	5 mg
Thiamin HCl	5 mg

(continued overleaf)

Nicotinamide	5 mg
Calcium pantothenate	5 mg
Cyanocobalamin	0.1 mg
p-Aminobenzoic acid	5 mg
Distilled water	1 l

This solution was filter sterilised, and added aseptically after autoclaving.

Medium PW. - large volumes of surface water were removed from Draycote reservoir and filter sterilised using 0.22 μ m pore filters (Millipore Ltd.). This was used without supplementation.

Medium PWP. - 100 mg of bacto-peptone (Difco) was added to 1 l of PW . After autoclaving, 10 ml of vitamin solution was added.

Medium PWPY. - 100 mg of bacto-peptone (Difco) and 100 mg of yeast extract (Difco) were added to 1 l of PW. After autoclaving, 10 ml of vitamin solution was added.

Medium PWB. - 100 mg of bacto-peptone (Difco) was added to 1 l of PW. After sterilisation, 20 ml of mineral base and 10 ml of vitamin solution were added.

Medium MB. - 20 ml of mineral base and 10 ml of vitamin solution were added to 1 l of PW.

Medium GMB. - 1 g glucose, 0.25 g $(\text{NH}_4)_2\text{SO}_4$, 0.07 g K_2HPO_4 were added to 1 l of PW. After autoclaving, 20 ml of mineral base and 10 ml of vitamin solution were added.

Medium GY. - 100 mg glucose, 100 mg yeast extract were added to 1 l of distilled water. 20 ml of mineral base and 10 ml of vitamin solution were added after autoclaving.

Medium GPY. - 150 mg bacto-peptone, 150 mg yeast extract, 250 mg $(\text{NH}_4)_2\text{SO}_4$ were added to 1 l of distilled water. 1 g glucose and 0.1 M phosphate solution were autoclaved separately, and added aseptically together with 10 ml of vitamin solution.

Medium Sm. - 50 mg bacto-peptone, 50 mg yeast extract were added to 1 l of distilled water. After autoclaving, 20 ml mineral base, 0.5 ml trace elements, 10 ml of vitamin solution were added together with phosphate buffer to a final concentration of 5 mM.

Medium Sg. - 2 g bacto-peptone, 2 g yeast extract, 1 g glucose were added to 1 l. of distilled water. 20 ml mineral base, 0.5 ml of trace elements, 10 ml of vitamin solution and 50 ml of 0.1 M phosphate buffer were added, after autoclaving the basal medium.

Medium M. - 1 g Na_2HPO_4 , 3 g KH_2PO_4 , 1 g $(\text{NH}_4)_2\text{SO}_4$, 1 g NaCl, 20 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg CaCl_2 , 1 g α -amino acids (Difco), 1 g yeast extract were added to 1 l. of distilled water. Glucose was added after autoclaving to a final concentration of 0.5% (w/v).

Medium C. - 20 ml of mineral base and 10 ml of vitamin solution were added to 1 l of distilled water. CH_3OH , $\text{CH}_3\text{NH}_2 \cdot \text{HCl}$ or HCOO.K were added aseptically, after sterilisation, to a final concentration of 0.01 M.

Medium HB. - This basal medium was prepared after Attwood and Harder (1972): K_2HPO_4 , 1.74 g; NaH_2PO_4 , 1.38 g; $(\text{NH}_4)_2\text{SO}_4$, 0.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.025 mg; $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 3.5 mg in 1 l of distilled water. 0.5 ml of trace element solution and 0.5% (v/v) CH_3OH were added aseptically after sterilisation.

Solid media. - When required, washed bacto-agar (Difco) was added at a final concentration of 1.5% (w/v).

(4) Enrichment, isolation and maintenance of budding and prosthecate bacteria

(a) Hyphomicrobium

Enrichment. - 100 ml of HB medium were placed in a 250 ml B19 Quickfit flask. 10 ml of a water sample from an oligotrophic water source was inoculated into the medium, the flask was sealed with a No. 37 Suba seal (William Freeman and Co. Ltd.) and gassed with O_2 free dinitrogen for 15 minutes. The flask was incubated statically in the dark at 30°C to allow for pellicle formation or on a rotary shaker (120 rev/min) to increase agitation (Attwood and Harder, 1972).

Isolation. - The flasks were regularly examined by phase contrast microscopy. After 4 weeks incubation, there was sufficient growth

for serial dilutions to be made, which were inoculated on to solid HB medium and into fresh HB medium. After a further 4 weeks incubation, individual colonies were picked, using a sterile toothpick, and streaked on to fresh medium. HB plates were incubated anaerobically in the dark, using a modification of the method of Westmacott and Primrose (1976) (see Section 3.II). Serial dilutions of 10^{-4} into liquid culture generally gave pure cultures of Hyphomicrobium.

Culture and maintenance. - Once isolated, Hyphomicrobium was maintained under aerobic and anaerobic conditions of growth, in liquid HB medium or on solid medium (Section 3.II). Stock cultures were routinely maintained in small glass vials, which were drop-frozen in liquid nitrogen and stored at -70°C , or on slopes of HB medium in 25 ml screw capped bottles which remained viable for several months.

(b) Caulobacter

Enrichment. - For the initial enrichment, 10 ml or 100 ml water samples were inoculated into a 250 ml wide necked flask with cotton wool plug, containing 100 ml of Sm medium. Flasks were incubated in the dark at room temperature or at 30°C , either statically to allow pellicle formation or on a rotary shaker for maximum aeration.

Isolation. - The liquid cultures were monitored by phase contrast microscopy, and after 4-5 weeks serial dilutions were plated on to solid Sm media. After a period of 2 weeks, colonies of interest were picked and repeatedly streaked on to fresh media to form a "patch". After a few weeks, individual colonies from these "patches" were streaked out, to isolate and purify the organism of interest, using a plate microscope (Olympus, Optical Co. Ltd.).

Culture and maintenance. - Once isolated, the Caulobacter cultures were grown in liquid or on solid Sg medium, under aerobic conditions, at 30°C . The cultures were maintained on slopes of Sg medium.

(c) Planctomyces

Enrichment. - For each enrichment, 100 ml of water sample was added to a sterile 150 ml beaker containing 10 mg of bacto-peptone (Difco), which was then left undisturbed in the dark at room temperature for

4-6 weeks (Staley, 1975). Alternatively, 10 ml of water sample was inoculated into 100 ml of PWP medium, which was incubated under the same conditions for 6 weeks or until there were signs of growth, e.g. turbidity or pellicle formation.

Isolation. - After this time, the enrichment cultures were serially diluted with filter - sterilised pond water; the dilutions were plated on to PWP medium and the plates incubated for a further 4 weeks at room temperature. Colonies present after this time were screened for budding, prosthecate or appendaged bacteria by phase contrast and electron microscopy. When detected, Planctomyces was isolated and purified by the same procedure outlined for Caulobacter, using GPY rather than Sm medium.

Culture and maintenance. - Planctomyces was maintained on GMB medium in liquid culture, under aerobic conditions, at 30° C or on solid medium.

(d) Mushroom shaped bacteria

The same screening procedure was used (see Caulobacter) using PWPY and GMB medium for the initial enrichment, and isolates were maintained on medium M.

(e) Ancalomicrobium and Prosthecomicrobium

Initial enrichment was as for Planctomyces (Staley, 1968). Colonies were screened, using phase contrast and electron microscopy. When detected, the colonies were picked with a sterile toothpick and patched on to medium PWPY, PMB or MB. After several weeks, individual colonies were streaked on to each of these media, to isolate and purify appendaged cells of interest. When isolated, these multiappendaged organisms were maintained on medium PMB or GMB.

(f) Pedomicrobium and Metallogenium

Enrichment. - These organisms were occasionally observed in PWP enrichments, especially if slides were suspended into the "enrichment" by means of cotton and metal clip, as a source of iron (see Attachment enrichments). Alternatively, 10 ml of water sample was added to 100 ml of medium C, in a 250 ml wide necked flask with cotton wool plug. This

was incubated in the dark at room temperature, for up to 6 months, or until there was sufficient growth.

Isolation. - A loopful of pellicle that formed was homogenised in 10 ml of medium C, and serial dilutions were plated out on medium C and HB. After 6 weeks incubation at 20° C, individual colonies were picked and streaked on to fresh media to give pure cultures of Pedomicrobium.

Culture and maintenance. - The cultures were maintained in HB and C medium, or on solid medium, with iron or manganese salt supplements.

Carbon source utilization

The ability of isolates of budding and prosthecate bacteria to use a variety of carbon compounds, as carbon and energy source, was tested using a medium containing 0.07 g/l K_2HPO_4 and 0.25 g/l $(NH_4)_2SO_4$, with 20 ml of mineral base and 10 ml of trace elements added after autoclaving to a litre of the salts medium. Carbon compounds were present as follows:-

Methanol	50 μ M
Methylamine HCl	20 μ M
Formate	20 μ M
Ethanol	0.1% (w/v)
Lactate	0.1% (w/v)
Propionate	0.1% (w/v)
Acetate	0.1% (w/v)
Citrate	0.1% (w/v)
Glucose	0.1% (w/v)
Fructose	0.1% (w/v)
Sucrose	0.1% (w/v)
Galactose	0.1% (w/v)
Glutamate	0.1% (w/v)

(5) Microscopy

(a) Light microscopy

All enrichments were routinely observed under phase contrast, using either a Leitz Orthoplan microscope fitted with an Orthomat unit (Leitz Instruments, Ltd.) or an Olympus EHT microscope with a PMT camera system (Olympus Optical Co. Ltd.). Plates were studied, using an Olympus 'Model X-Tr' stereoscopic microscope fitted with a PMT camera system.

Photomicrographs were taken on Panatomic X (Kodak, ASA 32), which was developed in D19 developer (Kodak) for 3 minutes, and fixed in Hypan rapid fixative (Ilford). Photomicrographs were obtained, using Contrast ff developer (Ilford) and Ilfrobrom paper, grade 5 (Ilford). Slide cultures were routinely used to determine the mode of microbial growth and the physiological requirements of the organism being studied. Slides and coverslips were sterilised, two drops of the appropriate molten nutrient agar were pipetted on to a microscope slide, and the agar was covered with a coverslip to give a thin, even layer of agar on the slide. After 5 minutes, the coverslip was removed, and a trough was cut through the agar, to ensure a supply of oxygen. The agar was then inoculated with culture, and a fresh coverslip applied, the sides being sealed with a paraffin/vaseline mixture. Details for anaerobic slide culture preparation are given in Section 3. II.

(b) Transmission electron microscopy

All studies were made using an AEI Corinth 275 electron microscope with an accelerating voltage of 60 kV. Electron micrographs were taken on 70 mm Ilford Line Film, N4E50, which was developed in Ilford Phenisol and fixed in Kodafix. Prints were made using Kodak Bromide paper.

The preparations were either negatively stained or metal shadowed.

Negative staining

Sample suspensions were pipetted on to formvar coated grids, fixed in OsO_4 vapour for 5 minutes, and then dried down on to the grids, drawing off any excess moisture with filter paper. A drop of 1% (w/v) phosphotungstic acid (pH 7.0) was added, and immediately absorbed off with filter paper. Alternatively, a drop of 0.5% (w/v) uranyl acetate (pH 4.5) was added, and removed after 5 minutes, the grids in this instance were then washed by dropping distilled water on to them, and then thoroughly dried.

Shadowing

Samples were prepared as for staining, and shadowed at an angle of 45° to 60° to the table of a vacuum coating unit (Nanotech Thin Films Ltd.) with gold/palladium alloy.

Sectioning - Ultrastructural studies

The fixation and embedding procedures of Ryter and Kellenberger (1958) and Spurr (1969) were followed for thin sections.

Ryter and Kellenberger. - Cells were centrifuged and washed thoroughly using a microcentrifuge (Quickfit Instrumentation) and fixed in 1% (w/v) osmium tetroxide in Kellenberger fixative (Ryter and Kellenberger, 1958). The cells were resuspended in a bacto-agar mixture (nutrient media + 1.5% (w/v) bacto-agar), and then cut into 2 mm cubes. These agar blocks were then stained in 0.5% (w/v) uranyl acetate, dehydrated through a series of alcohols, transferred to propylene oxide, and finally embedded in Araldite. The resin mixture was Araldite resin (CY212), 4.9 g; dodecenyl succinic anhydride (DDSA) hardener, 4.9 g; di-butyl phthalate (DBT) plasticiser, 0.075 g; benzyl dimethylamine (BDMA) accelerator, 0.175 g. Blocks were polymerised at 60° C for 3 days.

Spurr. - This is a low viscosity epoxy resin, which has the advantage of allowing the cells to be spun out of the resin, thus avoiding the need to suspend the cells in agar. Cell samples were spun down and fixed in glutaraldehyde and OsO_4 at 4° C (Spurr, 1969). Cell concentrates were then washed, stained in 0.5% (w/v) uranyl acetate, and dehydrated through a series of alcohols. Cells were finally transferred to and embedded in the resin (ERL 426 epoxide, 10.0 g; DER 736 hardener, 6.0 g; NSA, 26.0 g; S-I, 0.4 g). Blocks were polymerised at 80° C for a minimum of 8 hours.

Sections were cut on a Reichert "Om U2" ultramicrotome, using a Leitz diamond knife.

Sections were post stained in 0.5% (w/v) uranyl acetate for 20 minutes followed by 0.1% (w/v) lead citrate in 0.1 M sodium hydroxide (made with CO_2 free water), for 5 minutes when required.

(c) Scanning electron microscopy

Preparation of samples

Sterile polycarbonate nuclear pore membranes of 0.1 μm pore diameter and 25 mm diameter (Shandon Southern, Ltd.), glass coverslips of 9 mm diameter (Chance Proper, Ltd.) and formvar

coated grids of 3.05 mm diameter (Agar Aids, Ltd.), were floated on the surface of water enrichments or bacterial cultures for periods of time ranging from 1 day to 24 weeks, as surfaces suitable for bacterial attachment (Barrett and Pendergrass, 1977). After this time, each of these "surfaces" was carefully removed by means of a sterile metal loop or pair of forceps and immediately placed in a small glass petri dish containing 10 ml of 1% (w/v) glutaraldehyde in 0.15 M phosphate buffer, pH 7.2 for 3 hours at 20° C, replaced by 3% (w/v) glutaraldehyde in 0.15 M phosphate buffer, pH 7.2 for 24 hours at 4° C. Alternatively the cells were fixed in OsO₄ vapour from a 2% (w/v) solution of OsO₄ in 0.15 M phosphate buffer for 5 hours (Amako and Umeda, 1977). Aqueous OsO₄ was not used as this caused distortion (Horridge and Tamm, 1968; Falk, Gifford and Cutter, 1971; Boyde, 1972; Boyde and Vesely, 1972; Woldringh *et al.*, 1977).

The fixed surfaces were then dehydrated by replacing the fixative with (a) acetone or (b) ethyl alcohol.

(a) Acetone. - The material was placed in 5 ml of 20% (v/v) acetone, in a small glass petri dish, which was placed on the shelf of the desiccator, along with a watchglass of anhydrous CaCl₂. The base of the desiccator was filled with 100% (v/v) acetone containing some anhydrous CaSO₄. The desiccator was pumped with a conventional water pump until the acetone boiled, and then the desiccator was sealed under vacuum and left overnight, (Cohen, Marlow and Garner, 1968). On opening the desiccator, the material was found to be in 100% acetone, and had been fully yet gradually dehydrated by this diffusion method, minimising distortions due to rapid dehydration (Crawford and Gonda, 1977).

(b) Ethyl alcohol. - The material was initially placed in 10% (v/v) alcohol for 15 minutes, followed by transfers into 20% (v/v), 30% (v/v), 50% (v/v), 70% (v/v), 90% (v/v), 100% (v/v) alcohol, each for 30 minutes, avoiding rapid dehydration which causes cell shrinkage (Anderson, 1951). Finally the ethyl alcohol was further

substituted with amyl acetate, again using graded transfers of 25:75 (v/v), 50:50 (v/v), 75:25 (v/v) and 100:0 (v/v) amyl acetate to alcohol (Boyde, 1972).

The material in 100% (v/v) acetone or amyl acetate was then secured in a metal boat (Polaron Equipment, Ltd.) which was placed in a critical point drying apparatus (Polaron Equipment, Ltd.). The acetone or amyl acetate was replaced by liquid carbon dioxide every 30 minutes, to allow for impregnation of the new fluid into the material. The temperature of the ambient fluid after 2 hours was then raised above its critical point, whereupon it loses its capacity to form two phases, and its surface tension vanishes. The ambient vapour was allowed to slowly escape, to avoid adiabatic cooling and consequent recondensation, leaving the specimen behind. The specimen boat was then removed from the chamber, and the specimens were mounted on 13 mm Aluminium microscope stubs (Polaron Engineering, Ltd.). Coverslips and electron microscope grids were attached to the stubs with silver conducting paint (Polaron Engineering, Ltd.), whilst nucleopore membranes were attached with Durafix adhesive (Rawlplug, Co. Ltd.).

Most biological specimens are poor conductors, and under an electron beam build up charge on their surface. To prevent this build up, and to increase conductivity, it was necessary to coat the samples with a thin film of conducting material, such as gold-palladium alloy. Coating was carried out in a high vacuum evaporator with rotary table (Nanotech (Thin Films) Ltd.). To ensure a uniform coat of thickness 10 nm, a rotary table with a tilting device was used (Crawford and Gonda, 1977).

Specimen viewing

Specimens were viewed on a Stereoscan electron microscope (Stereoscan Mark 2A, Cambridge Scientific Instruments, Ltd.). Photomicrographs were taken using a Pentax SP500 camera using FP4 Ilford (ASA 125). Films were developed in Acutol developer (Paterson Ltd.) and fixed in Hypam (Ilford).

(6) Autoradiography

To determine the viability of the freshwater "enrichments" during their laboratory incubation, traces of ^3H labelled one-carbon compounds were added to the "enrichments", in quantities that would not significantly affect the cell morphology or growth (Ramsay, 1974). Enrichments were maintained in the presence of $^3\text{H CH}_3\text{OH}$ (specific activity 50 mCi/mmol) at a final concentration of 10 nM for periods of time ranging from one week to 12 weeks (Rogers, 1969a). 0.5 ml samples were taken at intervals, fixed with 2% (v/v) formaldehyde in veronal buffer, pH 6.0 for 1 hour, washed three times in veronal buffer and a smear was made on a clean slide, which was then air dried. Slides were then coated with Ilford L4 nuclear track emulsion (Bogoroch, 1972), diluted 1:1 with water, maintained at 45°C , according to the method of Rogers (1969b). Exposure was for a period of 1 to 12 weeks at 4°C , in a light tight box.

Autoradiographs were developed in a 1:1 dilution of D19 developer (Kodak) with distilled water or in Microdiol X, at 20°C for 5 minutes, and fixed for 5 minutes in Kodafix (Ryter, 1976). Developed autoradiographs were observed as wet mounts under phase contrast, or were dried and then stained with methylene blue for 2-5 minutes and examined under bright field optics (Ramsay, 1974), using an Olympus phase contrast microscope with oil immersion. In ordinary light microscopy, silver grains appeared as black dots on pale blue bacteria (Ryter, 1976).

(7) DNA extraction, determination of base composition

Cells were harvested in late exponential phase by centrifugation and washed twice with saline ethylene diamine tetraacetic acid (EDTA), (0.15 M sodium chloride and 0.1 M EDTA, pH 8.0). The cells were suspended in 0.1 M Tris-EDTA-saline (TES), (0.1 M Tris, 0.015 M EDTA, and 0.1 M sodium chloride, pH 7.1), with 4 mg/ml lysozyme and 100 $\mu\text{g/ml}$ pronase and incubated for 3 hours

at 37⁰ C. Lysis of the cells was completed by the dropwise addition of 5% (w/v) solution of sodium lauryl sarcosinate until the suspension became highly viscous. DNA was purified by centrifugation to equilibrium at 10⁰ C in CsCl of mean density of 1.71 g/cm³ for 36 hours at 120,000 g (MSE Super Speed 65 Centrifuge). Fractions were collected via a hole pierced through the bottom of the centrifuge tube, the DNA being detected as the viscous fraction. The extracted DNA was dialysed against 0.1 x SSC (10 times concentrate contained 87.7 g/L sodium chloride and 44.2 g/L sodium citrate). Sample concentration and purity were checked by measurement of its A₂₆₀ and the ratio of A₂₈₀/A₂₆₀.

DNA from Micrococcus lysodeikticus and E. coli were used as standards.

Analytical ultracentrifugation was carried out on a Beckman Model E analytical ultracentrifuge. A solution of CsCl (1.71 g/cm³ density) was prepared with 0.1 M TES buffer, pH 7.1, containing up to 2 µg of each species of DNA to be tested, and was loaded into the 12 mm 4⁰ Kel F centrepiece of the centrifuge cell. Centrifugation of the sample was for 22 hours at 44,000 rev/min at 25⁰ C (Schildkraut et al., 1962). Photographs were taken using ultraviolet absorption optics (Mandel and Marmur, 1968) and the films examined by print reproduction or by microdensitometer scans (Microdensitometer 3CS, Joyce Loebel). The buoyant densities and guanine plus cytosine ratios were calculated by comparison with M. lysodeikticus (1.7310 g/cm³, 72.45% G + C) and with E. coli (1.7092 g/cm³, 50.0% G + C), using the method of Mandel et al. (1968).

Section 2. III Present Work

1. Microscopical survey of the microbial flora of oligotrophic waters

Introduction

The use of the electron microscope to detect microorganisms in suspensions of peat (Volarovich et al., 1970), soil (Nikitin, 1973) and freshwater (Poindexter, 1964; Nikitin and Kuznetsov, 1967; Staley, 1968) have shown that the microflora of these environments are now readily amenable to investigation on a routine basis. Such investigations have already led to the indentification of many new morphological forms of microorganisms and their subsequent isolation (Staley, 1968; de Bont et al., 1970; Whittenbury and Nicoll, 1971; Bauld and Staley, 1975).

The numerical taxonomy and ecology of oligotrophic bacteria has been previously studied (Staley, 1971; Mallory, Austin and Colwell, 1977) and has shown that the prosthecate bacteria account for less than 1% of the total microbial population. These studies, however, only considered free flowing water, despite the fact that earlier studies had clearly shown that the prosthecate bacteria are commonly found attached to détritius or microflora, as well as to inert structures in the oligotrophic environment (Henrici and Johnson, 1935; Hirsch and Rheinheimer, 1968; Paerl, 1973). The study by Staley (1971) was carried out in a polluted stream throughout a calendar year, and clearly showed that there was no consistent correlation between the frequency of prosthecate bacteria and total coliforms in the stream during the investigation, however no comparable study was made in a more oligotrophic environment, that is one that is known to favour prosthecate bacteria (Hirsch, 1974). This has been rectified, in part, by the study of Mallory et al. (1977) of non-eutrophic aquatic systems, where the concentrations of available nutrients is low. Hyphomicrobium, Hyphomonas and Pedomicrobium were isolated together with many organisms possessing sheaths, most of which were unidentified. The prosthecate bacteria comprised the dominant organisms in this study, however, as Hyphomicrobium was described as a Gram positive rod

showing an absence of stalk, and not expressing any degree of pleomorphism (cf. Buchanan and Gibbons, 1974), any conclusions drawn from this work as to the significance of the prosthecae must be considered with caution.

Although the budding prosthecae bacteria have been shown to be ubiquitous in nature (Hirsch, 1974), in general they are in the minority. The exception to this appears to be in oligotrophic environments where their morphological adaptability is a clear advantage. Adaptation to this environment is not an exclusive property of the budding bacteria, for certain other groups of micro-organisms also appear to be flexible in the ability to alter their phenotype to optimise their survival potential (McGroarty, Koffler and Smith, 1973).

Certain bacteria optimise their survival potential by means of improved motility. Bacterial flagella as organelles of locomotion enable bacteria to respond to changes in their environment (Berg, 1975, 1976). Bacteria alter course by changing the direction of rotation of their flagella, cells with polar flagella back up, cells with lateral flagella try a new direction at random. Vibrio alginolyticus, isolated from coastal seawater, has been shown to change flagellar organisation when cultivated in the presence of certain chemical agents from lateral flagella to polar flagellar tufts (de Boer et al., 1975); however, to date this has not been shown to be a general property of flagellated bacteria.

Fimbriae/pili are the other major class of non-prosthecae cellular extension which are frequently observed on the cell surface of many groups of bacteria, especially the enterics (Hodgkiss et al., 1976). Pili are often implicated in adherence to bacterial cells, but they are of uncertain function, (Ottow, 1975). Certain bacteria found in an oligotrophic environment utilise their fimbriae in such a way as to confer upon them the property of matrix support (Hodgkiss et al., 1976). The matrix is spun about the bacterium and functions to trap in its binding capacity, molecules in the environment.

A further morphologically distinct type of non-prosthecae extension has been described, which appears to be a rigid structure, termed a spine (Easterbrook et al., 1973, 1976). These spines consist of rigid tubes which expand at the base and attach to, but do not originate in, the cell wall

(Willison et al., 1976). The wall of the spine is smooth on the inside, but ridged on the outside, the ridges being helically arranged along the length of the spine to give a striated appearance (Easterbrook, 1973). The spines were first observed in a marine bacterium, with the general properties of a member of the Pseudomonodaceae, and closely resembled 'the new type of fimbriae' described by Ahrens and Moll (1970).

Gas vacuoles have been reported to occur in a few species of bacteria and blue-green algae from aquatic environments (Houwink, 1956; Cohen-Bazire et al., 1969; Hirsch and Pankratz, 1970; Konopka et al., 1975; Walsby, 1977), appearing as bright, refractile bodies under the light microscope. Van Ert and Staley (1971a, b) have expressed the view that gas-vacuolated bacteria are common inhabitants of lake waters and attribute the fact that so few have been isolated to the inability of these bacteria to form gas vacuoles or even to grow in 'typical' laboratory media. The gas vacuole can be interpreted as a cellular device which enables its possessors to regulate their vertical position in an aqueous environment (Walsby, 1972). The heterotrophic freshwater bacteria Prosthecomicrobium pneumaticum and Ancalomicrobium adetum (Staley, 1968) possess gas vacuoles sufficient to render them buoyant at all stages of growth (Walsby, 1976). Both these strains are non-motile. Other strains from both genera are motile and do not possess gas vacuoles (Staley, 1968), possibly because their motility renders such structures unnecessary. As well as having a buoyancy role, it has also been suggested that gas vacuoles might perform a light shielding function (Cohen-Bazire et al., 1969; Walsby, 1972). Houwink (1956) pointed out that the presence of gas vacuoles results in an increase in the ratio of cell wall to cytoplasm volume, which would theoretically bring about an increase in the rate of exchange of substances by the cell; however, recent studies have been unable to demonstrate this (Walsby, 1975).

As has already been mentioned, the prosthecae bacteria represent a remarkable group of bacteria, only a few of which have been isolated and grown in pure culture (Zavarzin, 1960; Poindexter, 1964; Staley, 1968). Proposals for the function of prosthecae have been varied. When the caulobacters were first observed, their prosthecae were interpreted as stalks which served to anchor the cell to substrates; however, this anchoring is mediated by the secreted holdfast, located at the stalk tip, rather than by the stalk itself (Pate and Ordal, 1965). One obvious consequence of their

presence on the cell is to decrease the rate of sedimentation (Poindexter, 1964; Shapiro, 1971). Since most prosthecae bacteria are immotile in the stalked stage of their life cycle, the primary function of the prosthecae has been suggested to be one of buoyancy. In certain of the prosthecae, namely Hyphomicrobium and Rhodomicrobium, the prosthecae clearly have a reproductive function, but have also been shown to respond to nutrient concentrations in the same way as Caulobacter, thus suggesting that they might be nutrient uptake organelles (Lawrence and Dow, in preparation). Whether the reproductive function or the capacity for nutrient uptake is the primary function of the organelle is still not known.

Results and Discussion

'Static enrichment' studies

Throughout the duration of this project, a study was conducted to determine the incidence of heterotrophic bacteria in oligotrophic waters. Samples were routinely taken from several locations (Section 2. II) throughout the year, and scanned using transmission and scanning electron microscopes. Despite the fact that flowing water contained significantly less prosthecae compared to submerged inert surfaces, samples were generally taken just below the surface of the water, as this gave the most consistent numbers of prosthecae over a range of depths sampled (Table 2.1) and proved more convenient over the three year period of study, in agreement with previous studies (Hirsch and Rheinheimer, 1968; Marshall and Cruickshank, 1973; Dutka and Kwan, 1978). No significant change in the population was noted from any one sampling site; however, within the group of sites in the Lake District different water bodies clearly supported varying populations of microflora, reflecting the immediate environment, together with the effect of any agricultural, industrial or domestic pollution (Table 2.2).

Initially the prosthecae bacteria were considered to constitute up to 20% of the bacterial population, determined by counting in excess of 10^3 cells under the electron microscope (Figs. 2.5 and 2.6), a figure which did not appear to vary markedly with seasonal effects (Fig. 2.7). Debris and lysed cells were not considered; however, cell aggregates were considered as cell multiples. Phenotypic variations, which now appear prevalent among species of the multi-appendaged bacteria, were not considered at first, hence the initial figures for the relative proportions of prosthecae in the oligotrophic population are probably conservative (Lawrence and Dow, in

Table 2.1Prosthecate bacteria detected at various depths in Draycote WaterReservoir

<u>Depth (m)</u>	<u>Hyphomicrobium</u>	<u>Caulobacter</u>	<u>Multi-appendaged bacteria</u>
Surface	++++	+++	+
0.1	+++	+++	+
0.2	+	+	+
0.5	+	+	+
1.0	+	+	(+)
2.0	(+)	(+)	(+)
5.0	—	—	—
10.0	—	—	—
20.0	—	—	—
30.0	(+)	—	—
50.0	(+)	—	—

Scale + → ++++ abundant

— absent or not detected

Samples were examined by electron microscope, within two hours of
being collected.

Table 2.2

Variations in bacterial population with respect to location -
the effect of agricultural, industrial and domestic pollution
on bacterial counts

	<u>Sample site</u>	<u>TVC/ml</u>	<u>Heterotrophic prosthete VC/ml</u>	<u>%</u>
1.	Ennerdale	7.2×10^3	8.8×10^2	12.2
2.	Thirlmere	9.8×10^3	8.9×10^2	9.08
3.	Ullswater	5.8×10^4	3.9×10^3	6.72
4.	Haweswater	1.6×10^4	7.4×10^2	6.2
5.	Wise Een Tarn	4.3×10^3	6.9×10^2	16.0
6.	Three Dubbs Tarn	9.7×10^2	2.3×10^2	23.71
7.	Moss Eccles Tarn	5.8×10^3	7.8×10^2	13.4
8.	Windermere North Basin	8.2×10^3	1×10^2	1.21
9.	Windermere South Basin	9.0×10^3	3.5×10^2	3.88
10.	Esthwaite	1.4×10^4	2×10^2	1.42
11.	Coniston	9.3×10^3	1×10^2	1.07

Samples were plated out on GPY plates and incubated in the dark at 30° C for four weeks.

For sample sites - see Map, Fig. 2.3.

TVC = total viable count.

VC = viable count.

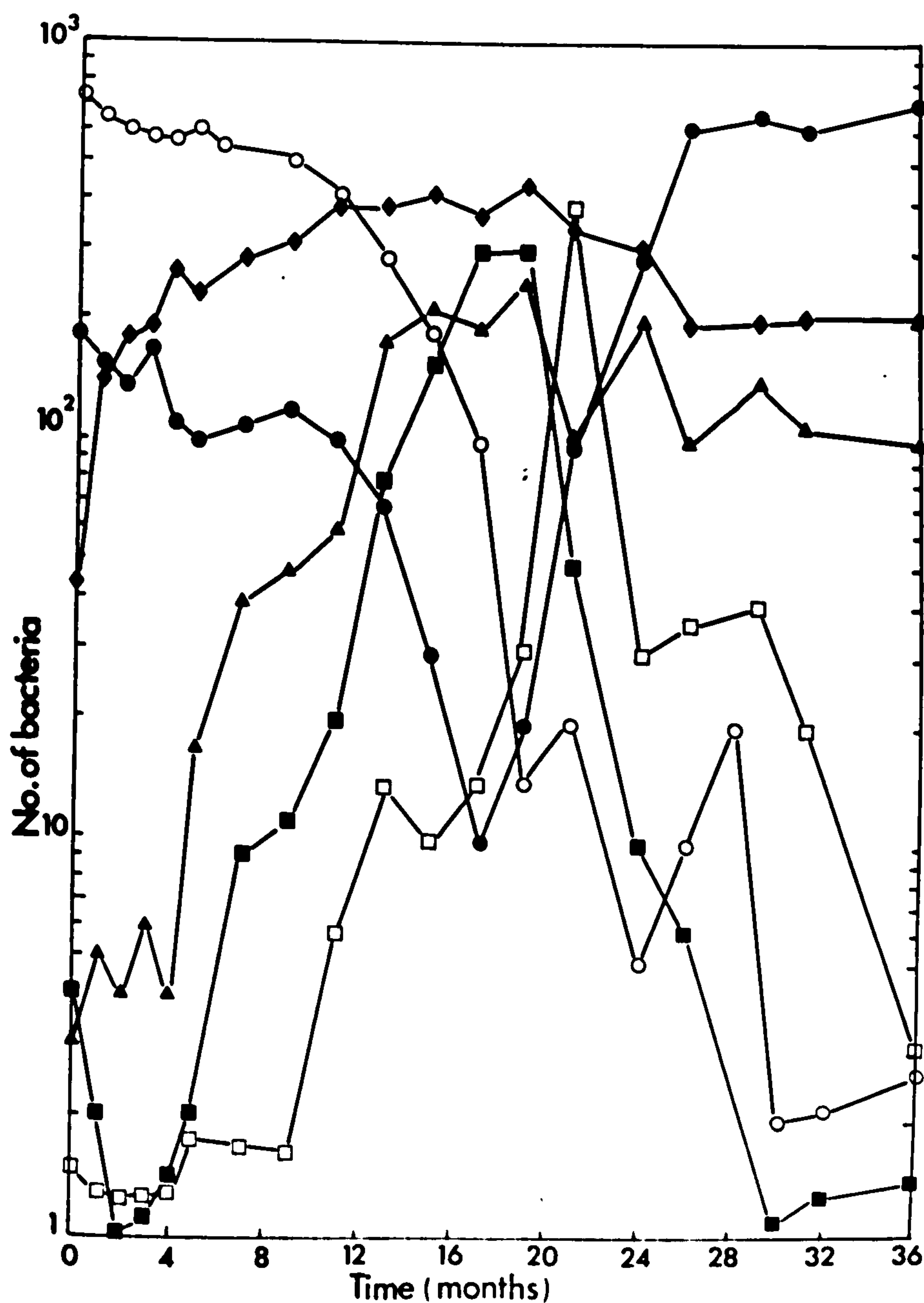


Fig. 2.5 Population graph of a Draycote Water Reservoir sample over a three year period of incubation (static enrichment). Approximately one thousand cells were counted on an E.M. grid, proportional numbers plotted against time.

<u>Hyphomicrobium</u>	—●—	Multiappendaged cells	—▲—
<u>Caulobacter</u>	—◆—	Gas vacuolated rods	—□—
<u>Planctomyces</u>	—■—	Others (rods, cocci, spirilla)	—○—

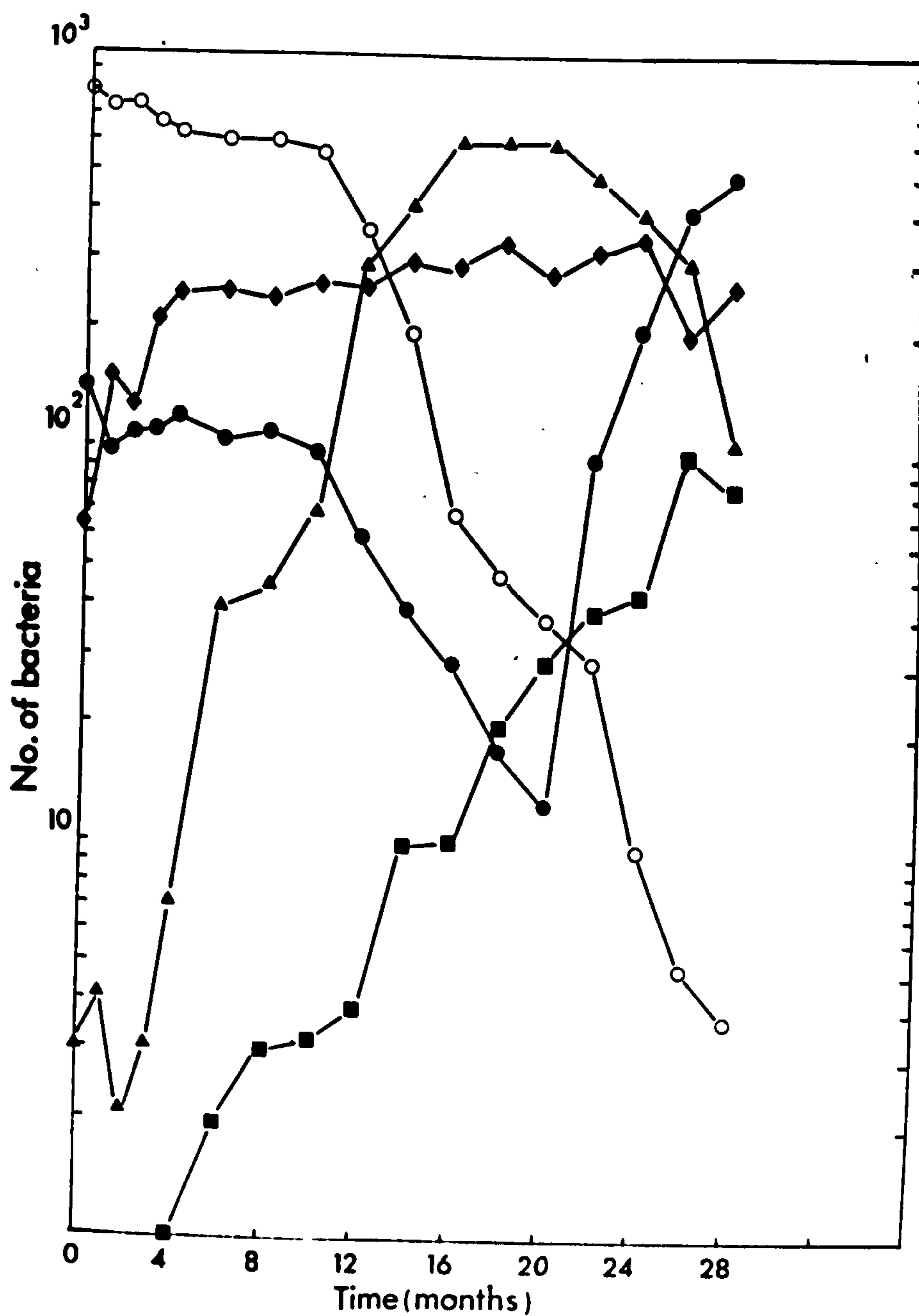


Fig. 2.6 Population graph of a Haweswater sample over a two year period of incubation (static enrichment). One thousand cells were counted on an E.M. grid, proportional numbers plotted against time.

Hyphomicrobium —●—

Caulobacter —◆—

Planctomyces —■—

Multiappendaged cells —▲—

Others (rods, cocci, spirilla) —○—

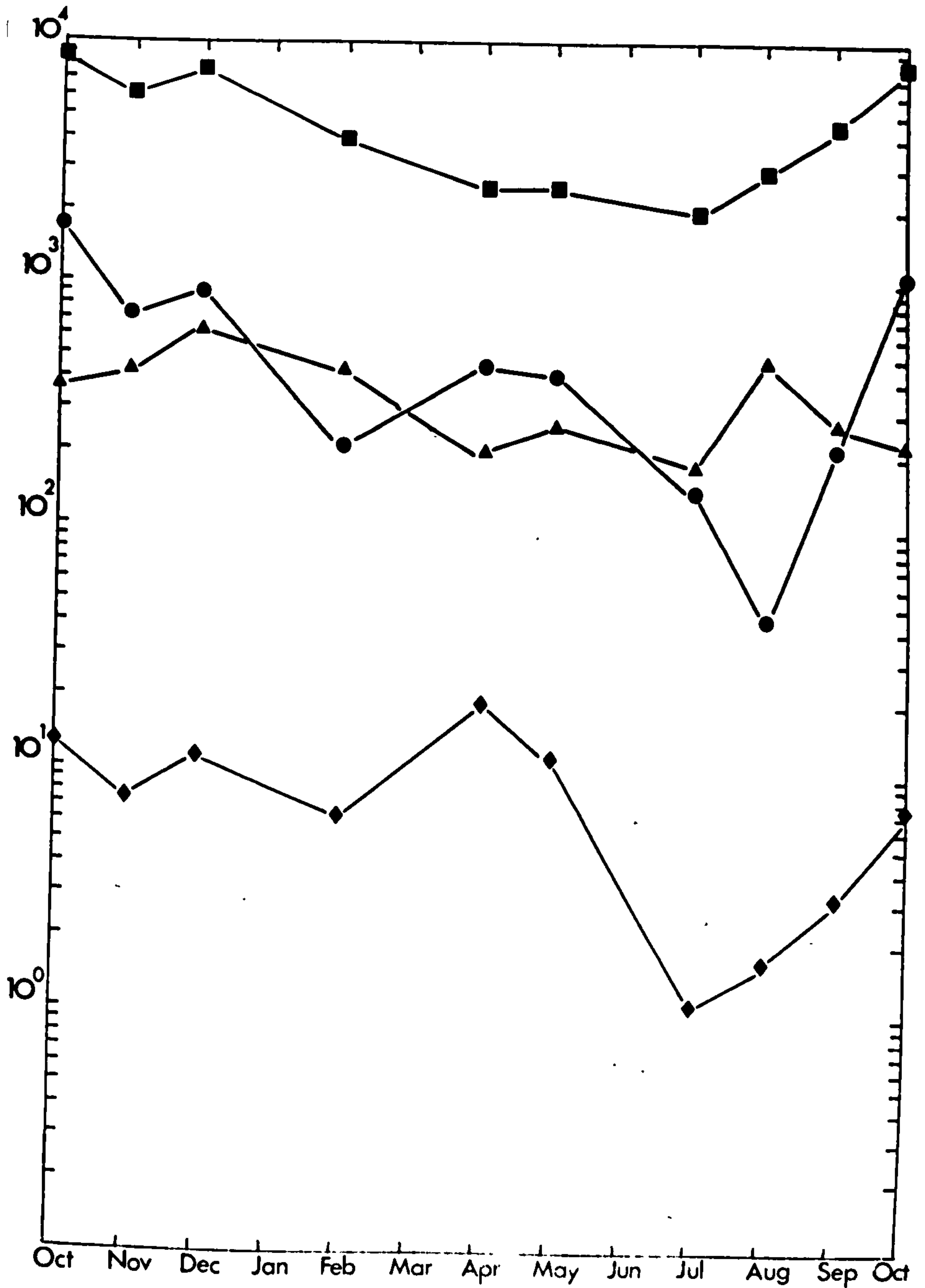


Fig. 2.7 Variable counts of bacteria from Draycote Reservoir over a twelve month period, as determined by plate counts and electron microscopy.

Total viable count	—■—
<u>Hyphomicrobium</u>	—●—
<u>Caulobacter</u>	—▲—
Multiappendaged cells	—◆—

preparation). Furthermore this figure agrees with the study of Mallory *et al.* (1977) although that particular study did not note the detection of Caulobacter sps., ubiquitous to oligotrophic environments.

To extend the study, water samples were collected and maintained as 'static enrichments' (Section 2.II). It was with these 'static enrichments' that some hitherto undescribed observations were made. Samples were regularly removed from (a) air-water interface, (b) middle of the body of water and (c) bottom of the glass flask, scanned for cell types, and these were then compiled into 'population' groups (Figs. 2.5 and 2.6). Over the three year period the bacterial cell types in the cell population varied considerably, the prosthecate bacteria eventually becoming the predominant cell type. After three years, samples could still be removed and plated out to give rise to a variety of colony types. Viability was also shown by the ability of cells, many bound in complex matrix formations (Fig. 2.8) to take up ^{14}C labelled methanol, added to the static enrichments at low concentrations (Section 2.II). Considerable care had to be employed as the resolution of autoradiography using the light microscope with prokaryotic cells is poor, and it became difficult to remove unincorporated label from the matrix without excessive washing, which disturbed the multicellular matrices. It does, however, permit the study of the incorporation of nutrients by such a population of cells (Fig. 2.9).

Whilst scanning the 'static' enrichments for population variations, it became quite clear that in an environment devoid of any renewed nutrients, cells would have to be adaptable in order to survive. In the natural environment, the survival capabilities of microorganisms are not so tested, because there is a constant renewal of nutrients, however low their concentration, and so it would be fair to say that, to a point, the 'static' enrichments are artificial in the same way as selective media, in this case selecting for organisms which can adapt to the condition of 'starvation'.

As has already been mentioned, some bacteria optimise their survival potential by certain morphological characteristics, e.g.

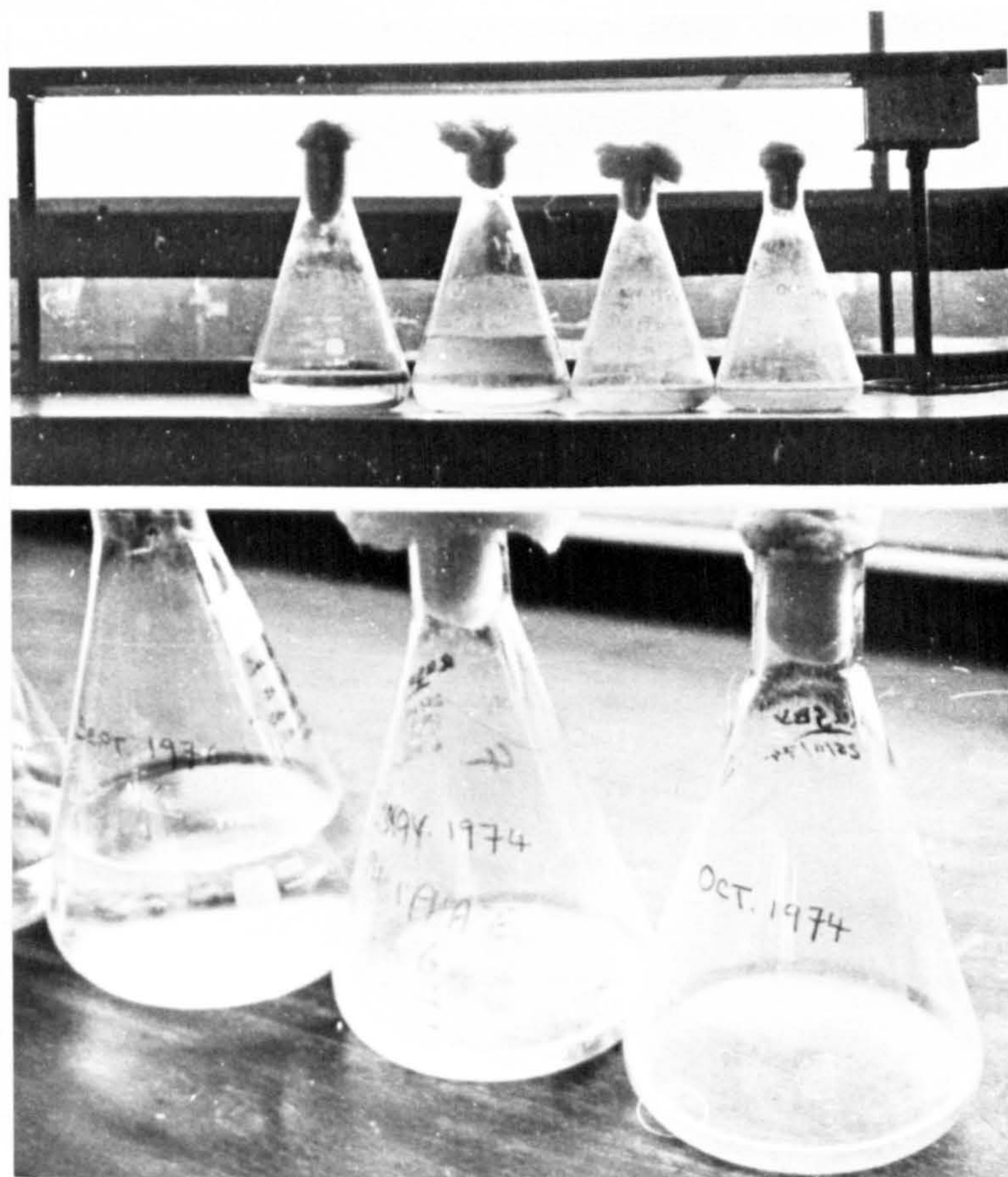


Fig. 2.8 Static enrichment flasks, left undisturbed for periods of time up to three years; no nutrients were added. Turbidity of flasks (Oct. 1974 cf. Sept. 1976) was due to multicellular complexes forming as matrices as the water 'aged'. (Normally cotton wool wad was covered with foil; flasks facing north light, no direct sunlight.)

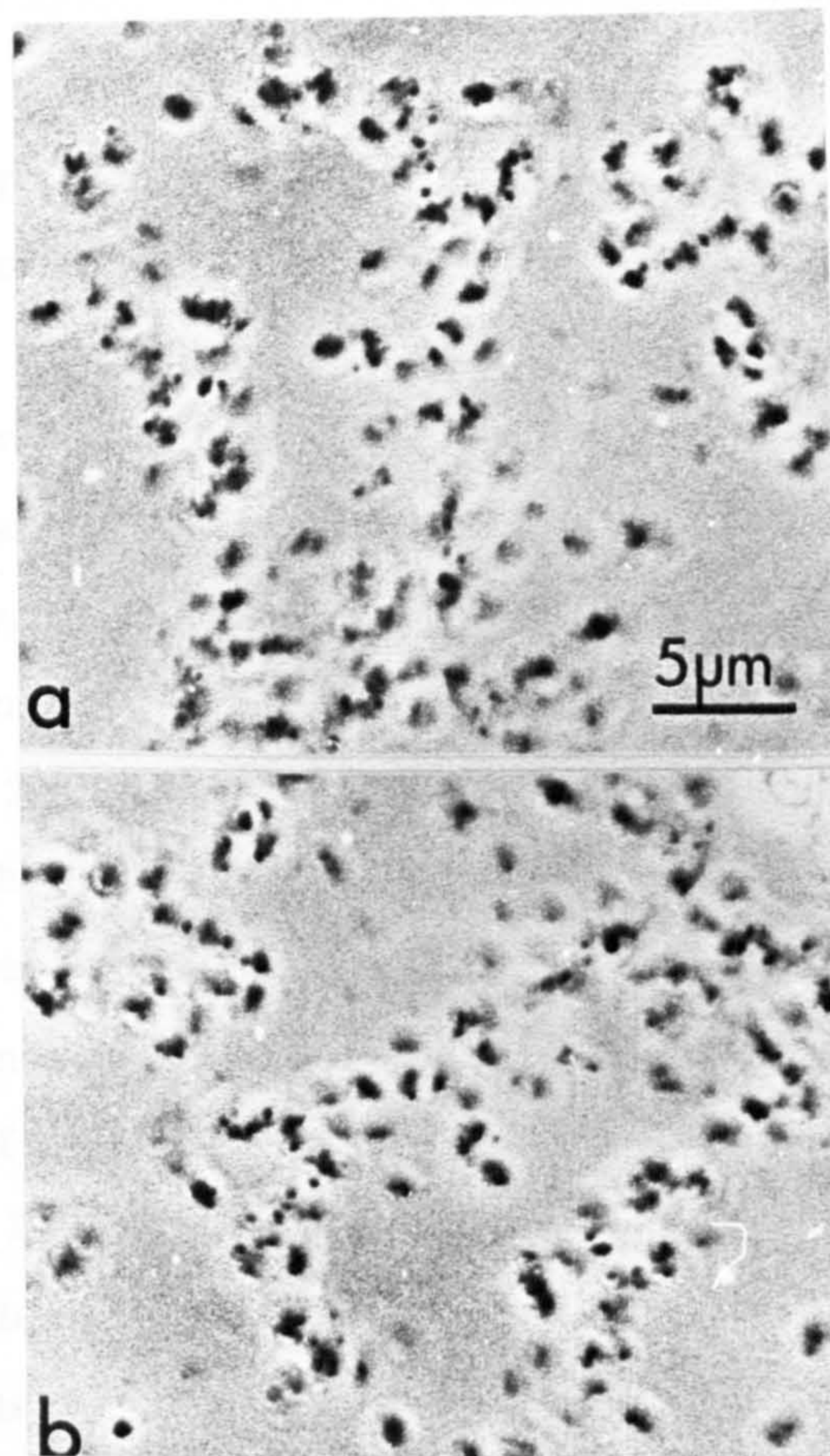


Fig. 2.9 Autoradiographs of matrix formations from 'static' enrichment flasks. Silver grains appeared to be associated with the cell bodies and not with the prosthecae which are poorly resolved, implicating the former with carbon assimilation into cell constituents.

fimbriae, gas vacuoles. As the 'static enrichment' flasks were 'starved' of nutrients over the three year period, bacteria possessing such characteristics appeared to be able to withstand the 'starvation' conditions to a greater extent, compared to other bacteria incapable of adapting their morphology. This phenotypic flexibility appeared to optimise the survival potential of these groups of bacteria over the population as a whole.

Bacterial cells, frequently encountered in these enrichments after prolonged incubation, possessed characteristic flagellar arrangements (Fig. 2.10). One can speculate that flagellation may provide these bacteria with a selective advantage over non-motile bacteria, in a nutrient poor environment. Other groups of bacteria possessed arrays of fimbriae about the cell body (Fig. 2.11). The fimbriae confer upon the cell body an additional layer, which may have a protective role or be a means of concentrating nutrients from the environment. The fimbriae of Planctomyces extend for many times the length of the cell body, as the environment becomes depleted of essential nutrients, and intertwine to form networks with adjacent cells (see Fig. 2.19a). Spined bacteria were also observed in these 'static' enrichments, bound by extracellular material (Fig. 2.12). What advantage the spines confer upon the cell is not known, but it seems reasonable to regard these acellular structures as having a supportive function.

Fig. 2.13 illustrates some of the more unusual bacteria which possess cellular extensions; however, although (b) resembles Prosthecomicrobium (Staley, 1968), (c) and (d) do not fit any known genus, and the cellular extensions in (a) could well be an artefact resulting from specimen preparation, i.e. surface tensions effects, as described by Klainer and Betsch (1970).

Routine surveys showed that these morphologically unusual bacteria were frequently observed in association with algae, diatoms, protozoa and higher organisms, as has been previously noted (Skuja, 1964; Paerl, 1976). Figs. 2.14 and 2.16b illustrate some of the associations observed. Employing a simple chemostat apparatus (Section 2.II), water sample containing these 'associations' were grown in continuous culture, under aerobic conditions, with a light intensity of 500 lux, for periods of up to six months. Up to dilution rates of $D = 0.3 \text{ h}^{-1}$, associations of bacteria with algae developed;

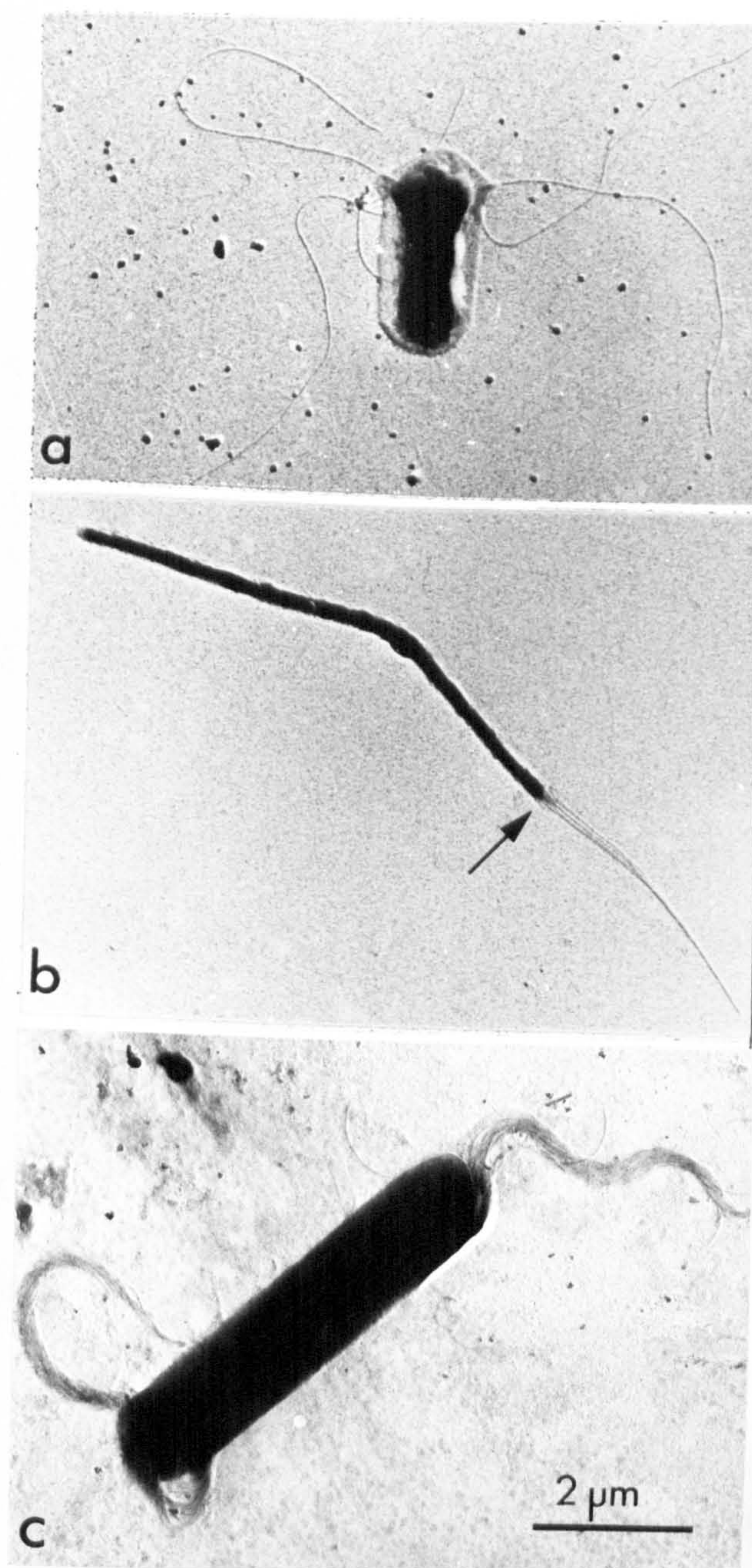


Fig. 2.10 Flagellated bacteria in oligotrophic waters.

(a) peritrichously flagellated rod.

(b) rod with polar tufts of flagella

(c) polar tufts (termed fascicles) of flagella (as described by Strength et al., 1971).

(Gold/Palladium shadowed).

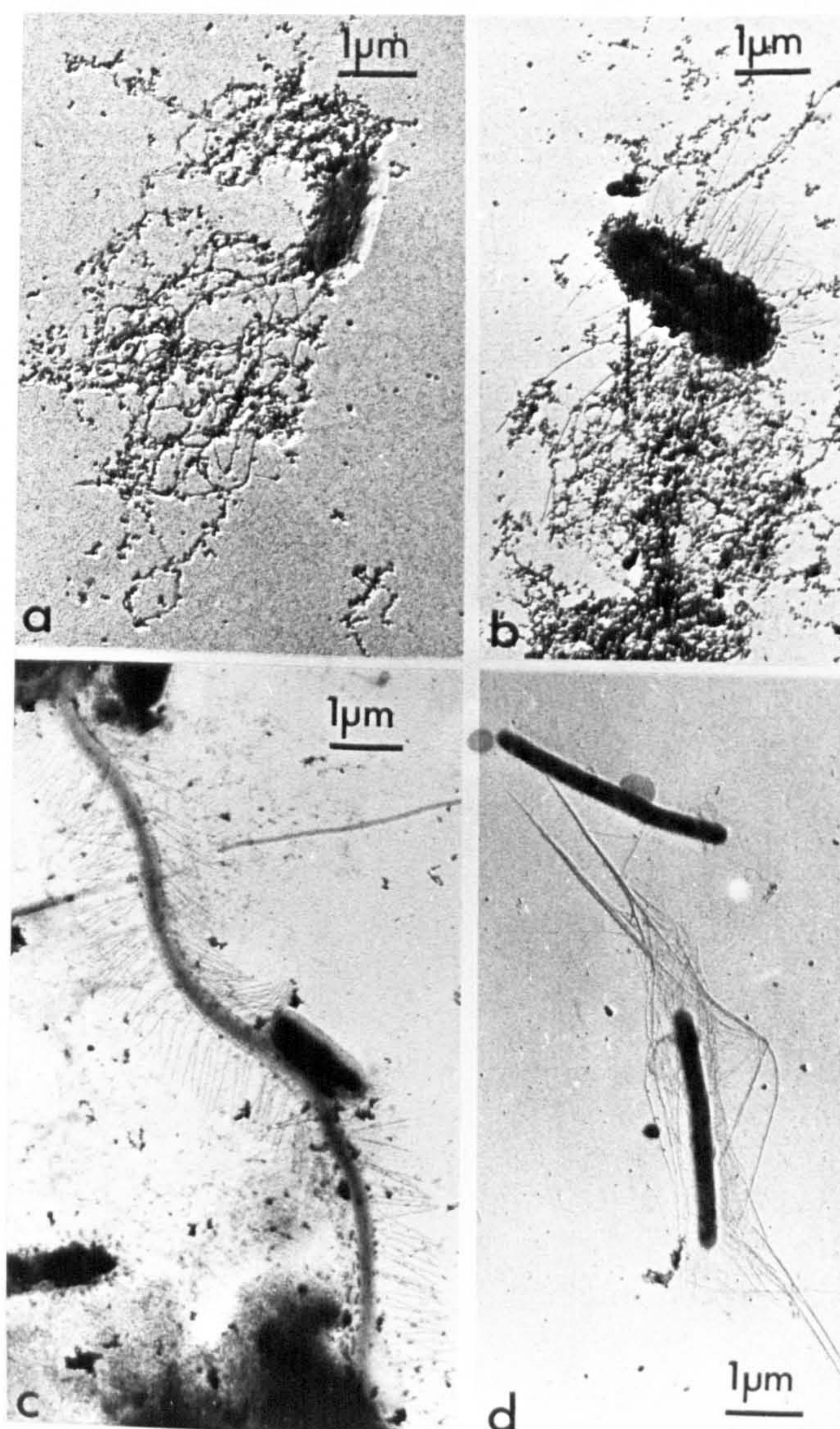


Fig. 2.11 Bacteria with arrays of fimbriae.

- (a) and (b) metal deposition associated with fimbriae
 - (c) fimbriae cover the total cell surface, conferring upon the bacterium a protective layer, capable of concentrating nutrients from the environment
 - (d) network of fimbriae about a cell
- (Gold/Palladium shadowed).

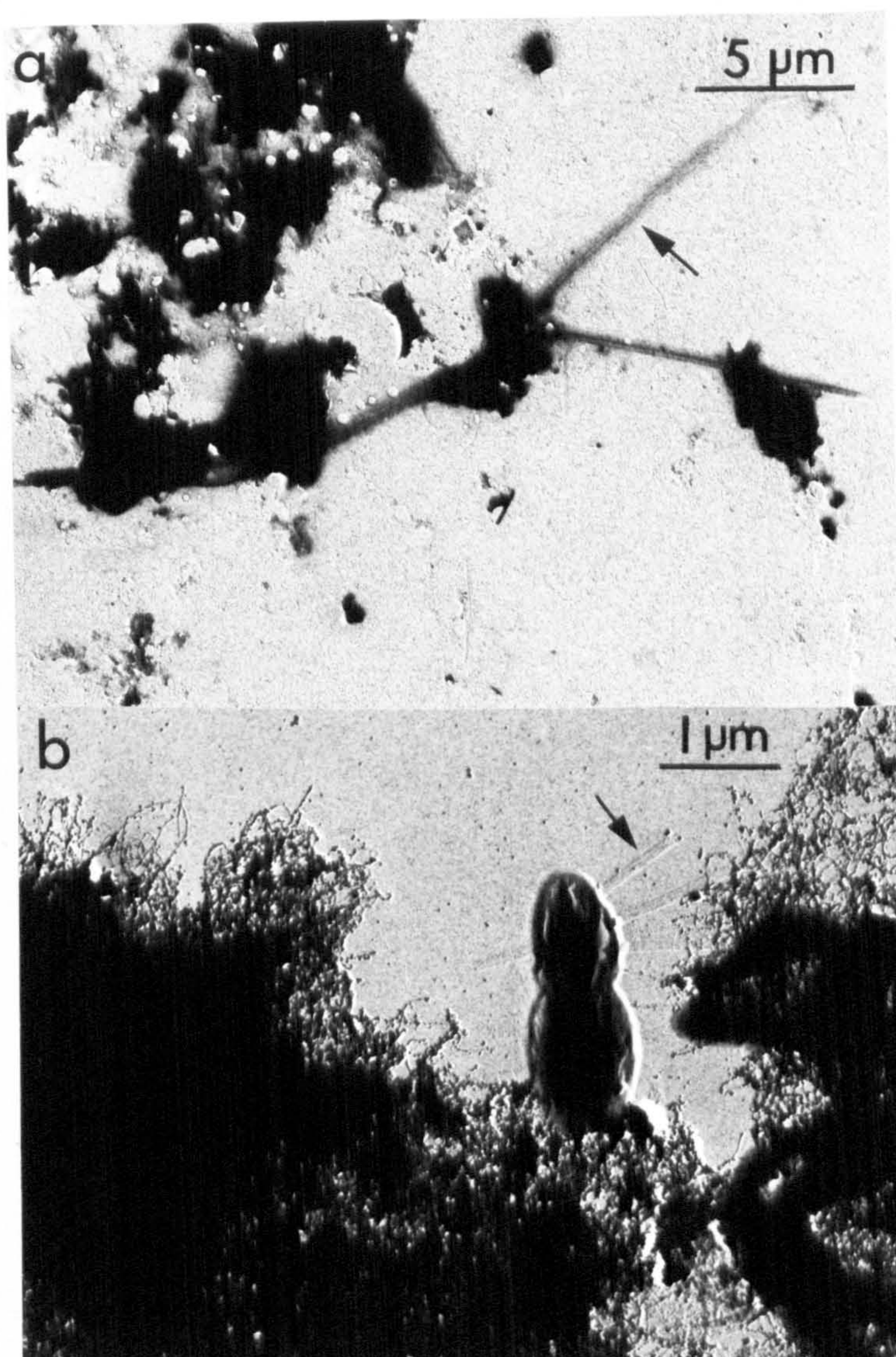


Fig. 2.12 Bacterial spines

(a) a bacterium with three spines extending from the cell body

(b) spined bacterium associated with extracellular material

(Gold/Palladium shadowed).

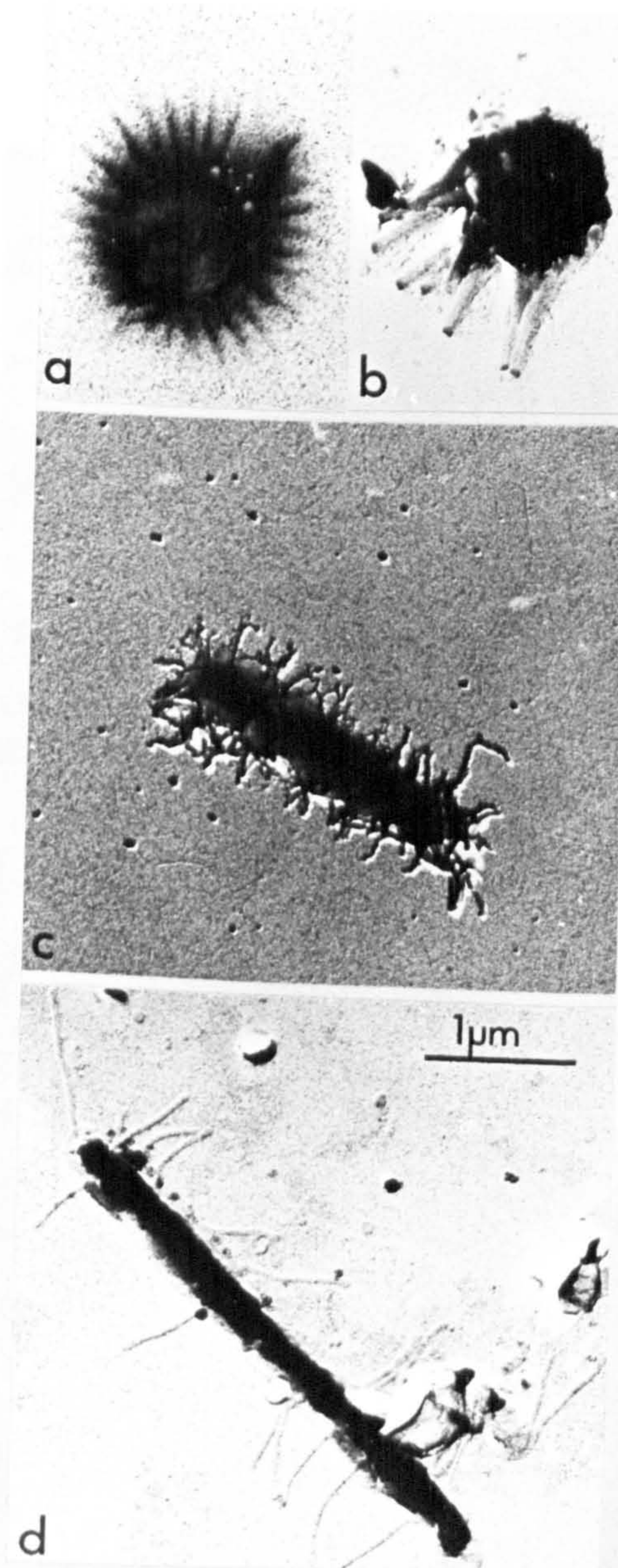


Fig. 2.13 Bacteria with extensions, cellular and acellular.

(a) coccoid cell with a 'prickly' periphery

(b) presumptive Prosthecomicrobium

(c) rod with short cellular extensions

(d) rod with fine extensions, their nature not determined from the micrograph

(Gold/Palladium shadowed).

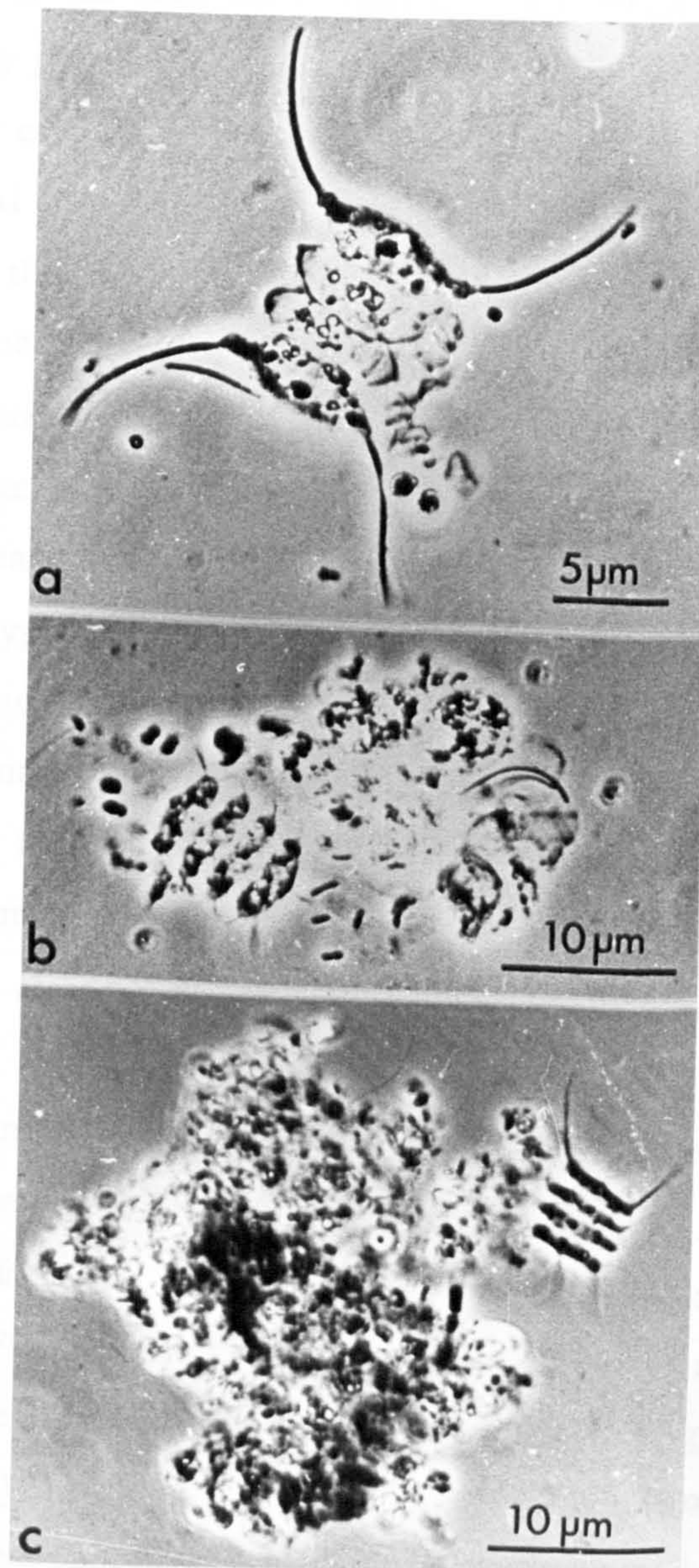


Fig. 2.14 Bacterial association with algae (light micrographs)

(a) and (b) associations in the natural environment

(c) bacterial/algal associations in a simple chemostat

(see Text).

however, the number of prosthecate bacteria, relative to the total population, did not increase significantly. Above the dilution rate of 0.3 h^{-1} , the culture washed out, preventing the system from being used to mimic the natural 'dynamic' situation in moving water, e.g. a stream, where it has been shown that bacteria adapt to their surroundings by producing extracellular polymers enabling them to adhere to other cells or inert surfaces (Cagle, 1974).

Microscopical observations showed that as the 'static' enrichments 'aged' over the three year period, the cell morphology became more varied as each cell type was, in effect, 'pushed to the limit', attempting to adapt to the starvation conditions. Obviously there were considerable biochemical rearrangements, but this study limited itself to phenotypic variations, as early chance observations under the electron microscope, of bizarre cell types in oligotrophic waters had implicated the prosthecate bacteria in the survival 'race' under starvation conditions.

Within a month of setting up the 'static' enrichments, pellicle formation at the air-water interface became readily visible. Examination of the pellicle showed many of the cells were organised in mixed populations, as cell aggregates, adhering together to form rosettes by means of holdfast structures (Fig. 2.15a) (cf. Fletcher and Floodgate, 1976). After a further month, many of the rod-shaped cells, which had been the major cell type when the water samples were originally obtained (Fig. 2.6) had disappeared; budding rod cells were still prevalent, however, growing as sheets of cells (Fig. 2.15b). Over the first eighteen months, Caulobacter sp. rapidly increased in number, ultimately becoming the dominant cell type (Fig. 2.15b). As the nutrients became more limiting, the length of the stalk increased dramatically (Fig. 2.17); however, the number of crossbands along the length of the stalk appeared to be random (Fig. 2.17c) (cf. Staley and Jordan, 1973).

After twelve months of incubation, as 'static' enrichments, the fresh-water samples became dominated by prosthecate bacteria, which either produced tangled networks of cells with cellular stalks or were attached to inert structures by means of their holdfasts (Fig. 2.16). The level of nutrients in the water was probably maintained at a low concentration by

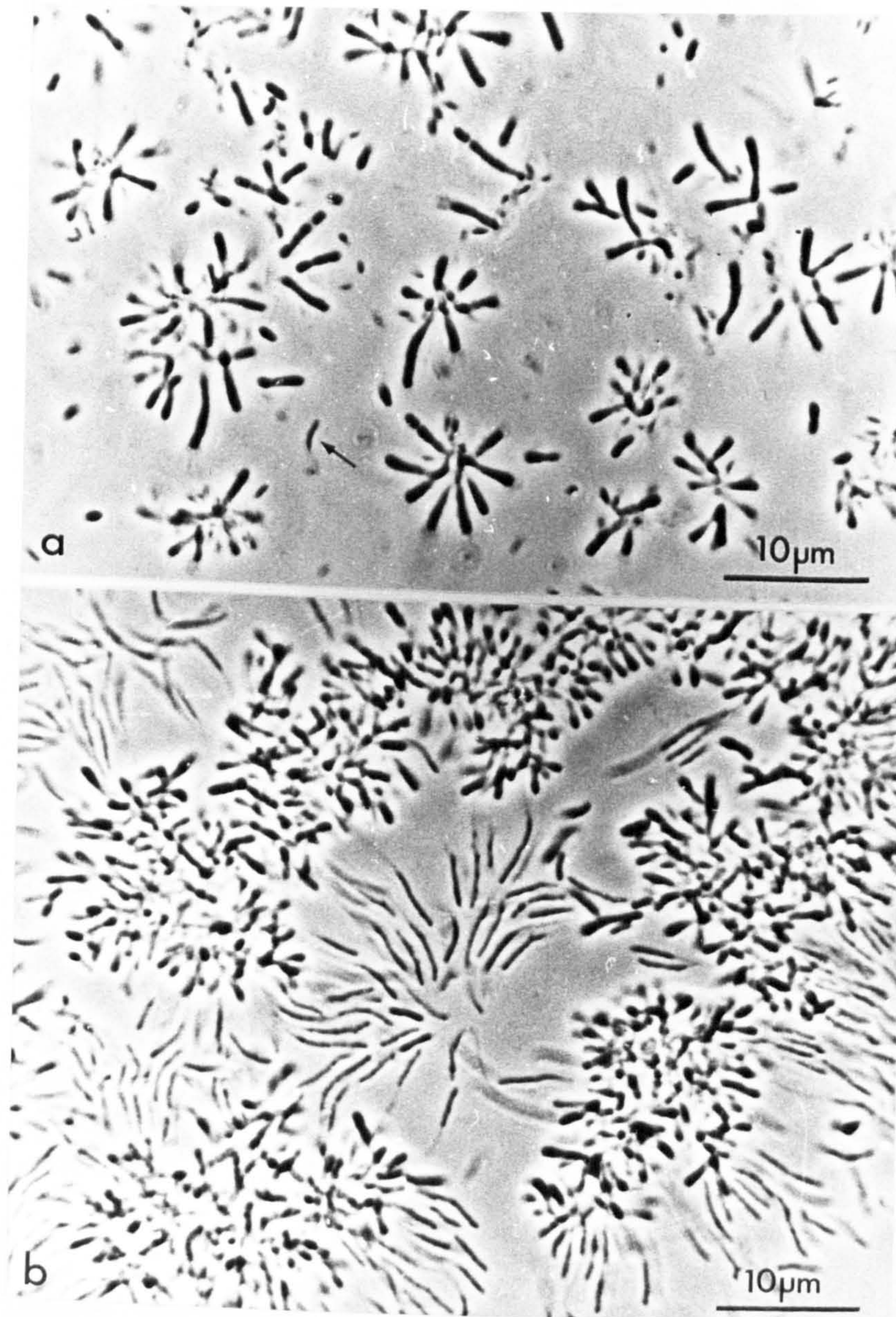


Fig. 2.15 Cell-cell interactions at air-water interface (light micrographs).

(a) Budding bacteria frequently present as rosettes, after one month's incubation. Occasionally Caulobacter were present (arrowed).

(b) After two months' incubation, Caulobacter, attaching to other bacteria, by means of their holdfast, were prevalent in the pellicle formed.

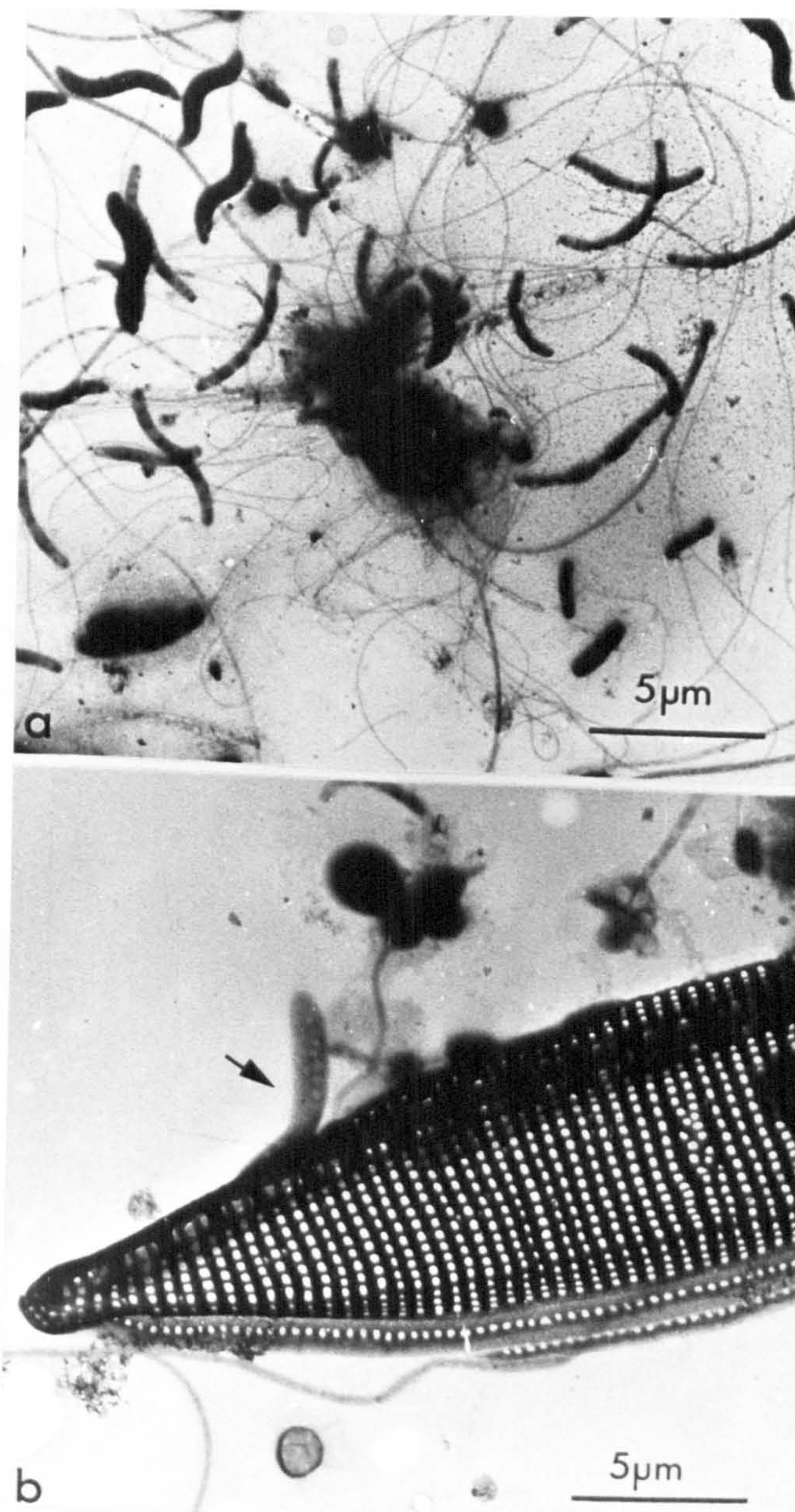


Fig. 2.16 Cell-cell interactions

(a) Cellular complexes developed; commonly the prosthecate bacteria formed networks in which other cells were trapped, or bacteria adhered (after one year's incubation as a static enrichment).

(b) Bacteria attaching to the surface of a diatom in the 'natural' enrichment (Draycote Reservoir, after one year's incubation).

(Gold/Palladium shadowed).

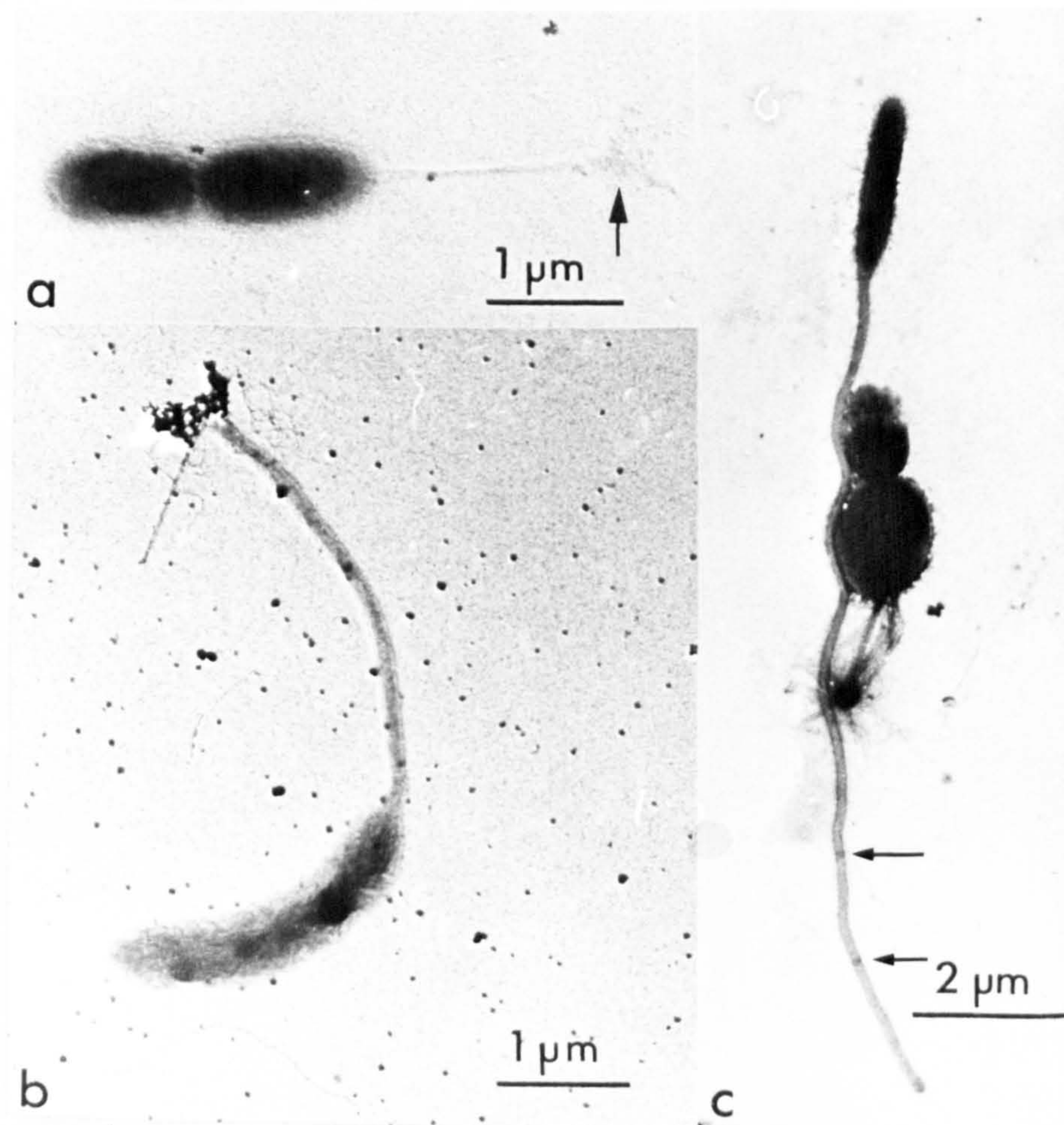


Fig. 2.17 Caulobacter

(a) and (b) are classic cell types with prominent holdfasts, from 'static' enrichments.

(c) Caulobacter with Planctomyces, adhering by means of its holdfast. Note random placement of crossbands along length of Caulobacter stalk. [(a) and (b) - gold/palladium shadowed, (c) negatively stained with phosphotungstic acid, PTA].

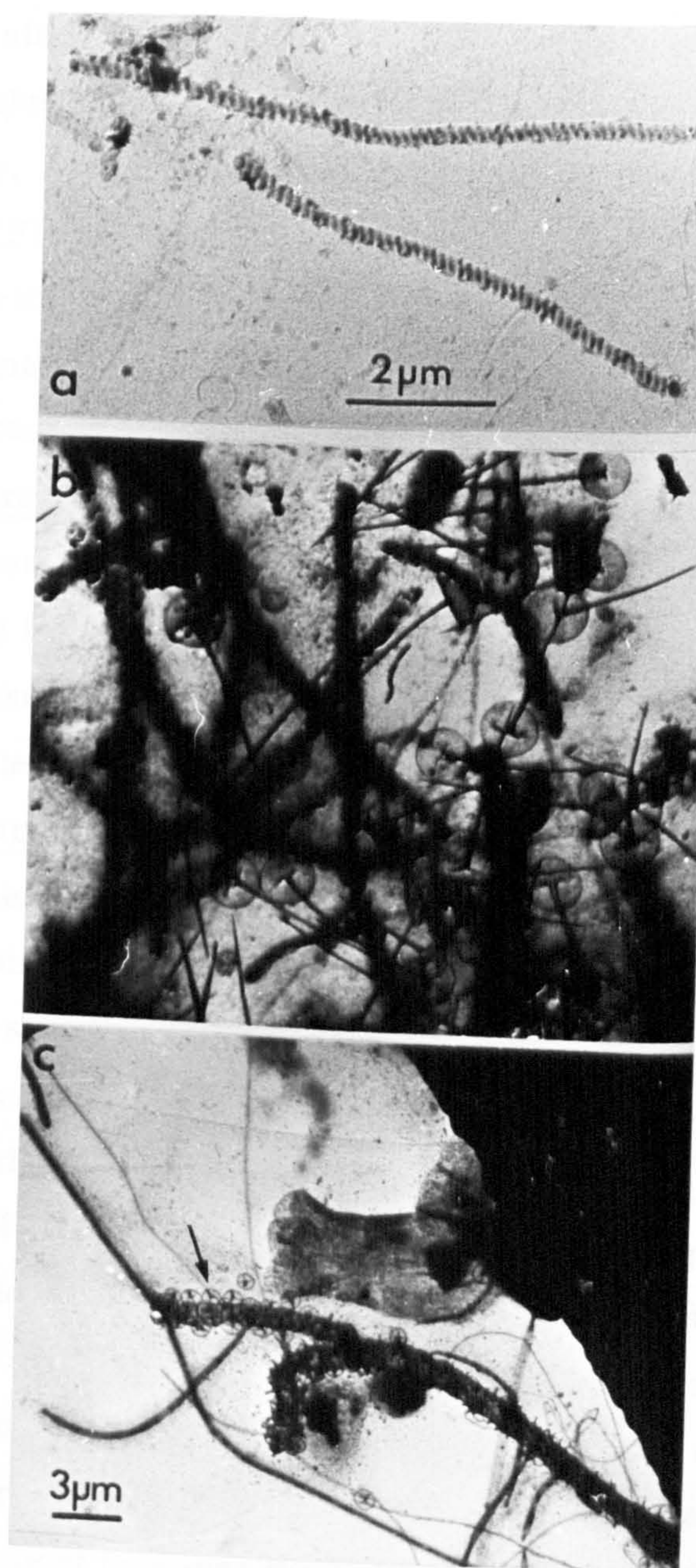


Fig. 2.18 Seliberia and 'umbrella'-shaped structures.

(a) Seliberia, showing prominent helical surface patterns.

(b) Scales from the colourless flagellate, Paraphysomonas vestita.

(c) Scales from Cyathoboda, another colourless flagellate (arrowed).

(Gold/Palladium shadowed).

the death of cell types unable to survive these incubation conditions, their autolysis maintaining a cycle of basic nutrients. As the enrichments became further devoid of essential nutrient, Planctomyces sp. became evident (Fig. 2.17), together with Seliberia and unusual 'umbrella-shaped' structures (Fig. 2.18). Seliberia (Aristovskya and Parinkina, 1963) remained in the enrichments throughout the three year period, but was never present in sufficient numbers for successful isolation. The skeletal structures resembled hatpin-like scales or 'umbrellas' and were the body scales of Paraphysomonas vestita (Stokes) De Saedeker, a colourless chrysophycean flagellate (Manton and Leedale, 1961) (Fig. 2.18b), which is a common freshwater flagellate. Fig. 2.18c illustrates a large lorica, probably belonging to a species of Dinobryon (Chrysophyceae), and small quadrate scales resembling those of a species of Cyathoboda, or another species of Paraphysomonas (Swale and Belcher, 1975). The Planctomyces sps. developed in good numbers (Fig. 2.6) and appeared to be well adapted to the environmental conditions, possessing an extensive pill network. Although the acellular stalk does not have a direct role in nutrient uptake, as in the case of Caulobacter, it enables the cell to attach to substratum by means of the holdfast, and the cell body can swing in an arc, increasing the 'accessibility' to the immediate environment (Fig. 2.19a) (cf. Hirsch, 1972). Motile cells are budded off the attached cell and presumably serve a dispersal role. The stalk of Planctomyces is not an integral cellular extensions, and this might explain why Planctomyces eventually declines in number, whereas Caulobacter, the more adaptable prosthecate bacterium, maintains constant numbers throughout the enrichment, by stalk elongation as the environment is 'starved', thus giving the cell an increased surface area relative to cell volume, so presumably enhancing nutrient uptake.

In the 'static enrichment' flasks, surviving rod shaped bacteria had developed heavy capsules (Fig. 2.19c) or were gas-vacuolated (Fig. 2.20b and c). The encapsulated bacteria were frequently associated with networks of prosthecae bacteria, whereas the gas-vacuolated bacteria were observed free in the aqueous environment.

After one year's incubation, the 'static' enrichments were composed essentially of Planctomyces, Caulobacter and the multi-appendaged bacteria, the gas-vacuolated rods dominating the population for a short period of time (Fig. 2.6). The multi-appendaged bacteria not only included the well documented Ancalomicrobium and Prosthecomicrobium (Staley, 1968), but also cell types, previously undocumented. Some of these are illustrated in Figs. 2.21, 2.22 and 2.23, and have been studied in detail (Section 2.III.2). The multi-appendaged bacteria were frequently associated with tangled networks of stalks from other prosthecae bacteria, e.g. Caulobacter, and cells with extensively fimbriated peripheries (Fig. 2.21a), some of which possessed acellular stalks, e.g. Planctomyces (Fig. 2.21b). At this time the multi-appendaged cells were still mainly characterised by their classic phenotype, although the 'knobbed' rod was now recognised as a phenotype variant in a non-expressed form (Fig. 2.24). Surprisingly, as the enrichments aged, the non-expressed form was still present, until at the end of the three year study the numbers of expressed and non-expressed forms of multi-appendaged bacteria were reduced to less than 10% of the total microbial population (Fig. 2.6).

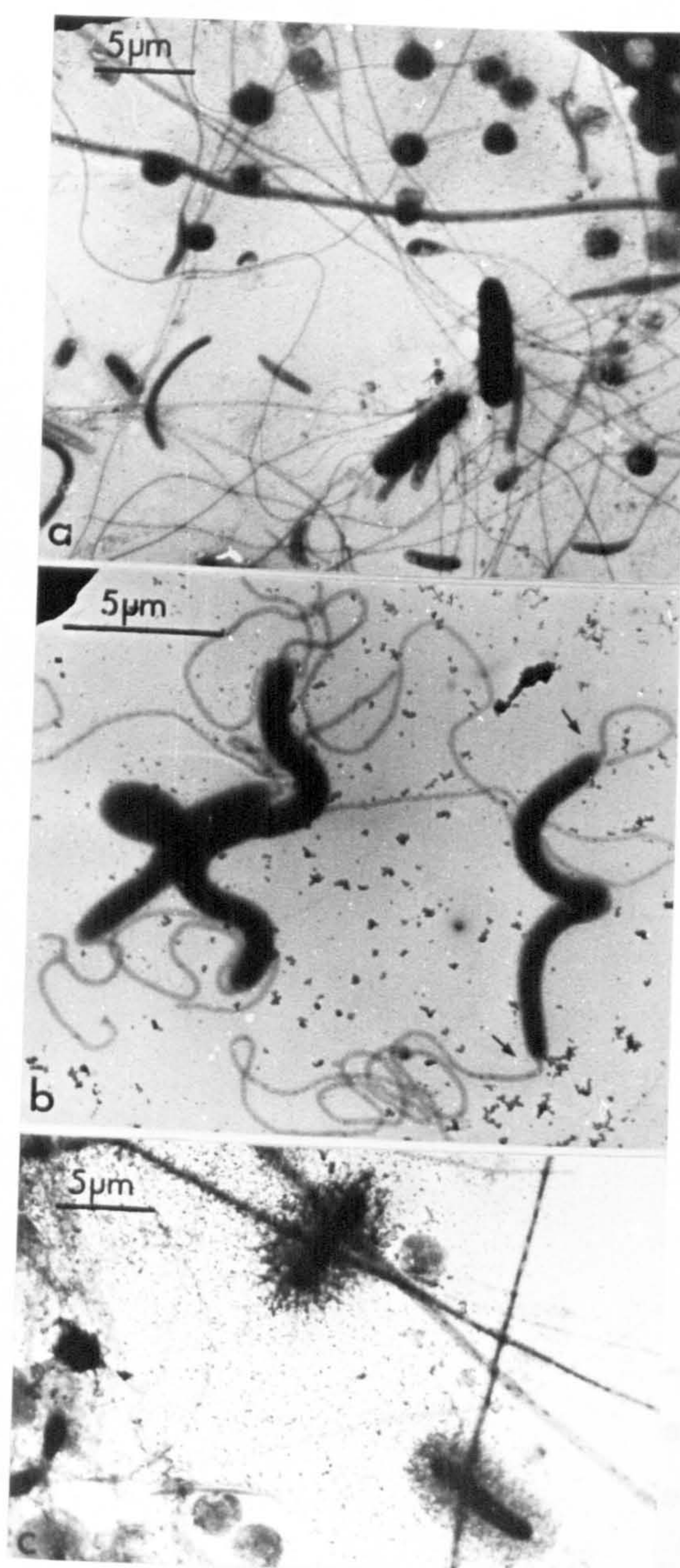


Fig. 2.19 Adaptation to the environment

- (a) Cellular networks, comprising of Planctomyces and prosthecate bacteria.
 - (b) Bacteria possessing stalks at both poles, which appear to be prosthecal.
 - (c) Rod cells surrounded by capsular material.
- (Gold/Palladium shadowed).

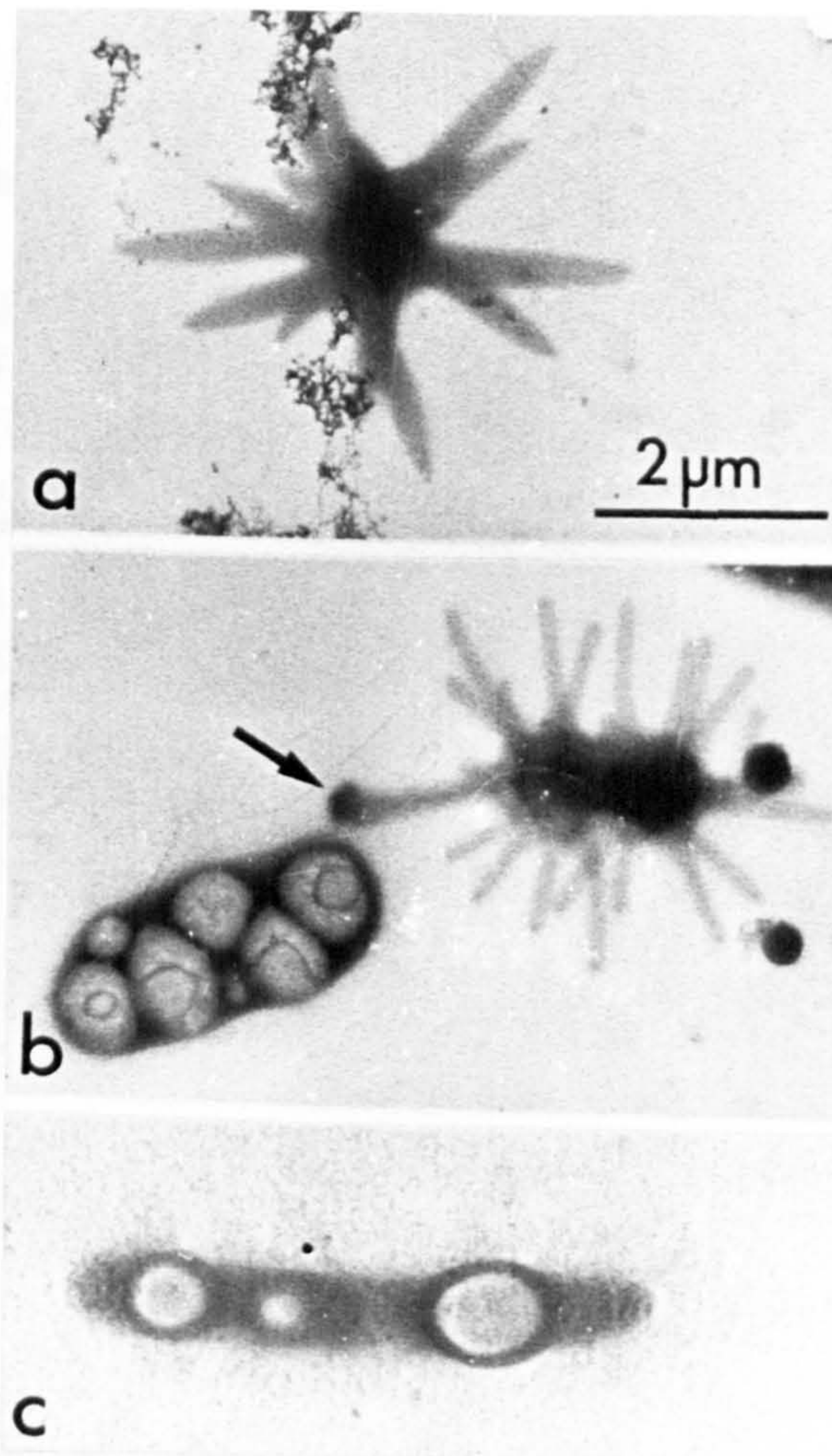


Fig. 2.20

- (a) Presumptive Prosthecomicrobium in metal deposits.
- (b) Presumptive Pedomicrobium, in the process of cell division, with swelling from one appendage (arrowed), together with gas vacuolated rod.
- (c) Gas vacuolated rod, with two prominent gas vacuoles, from Draycote Reservoir 'static' enrichment (12 months).

(Gold/Palladium shadowed).

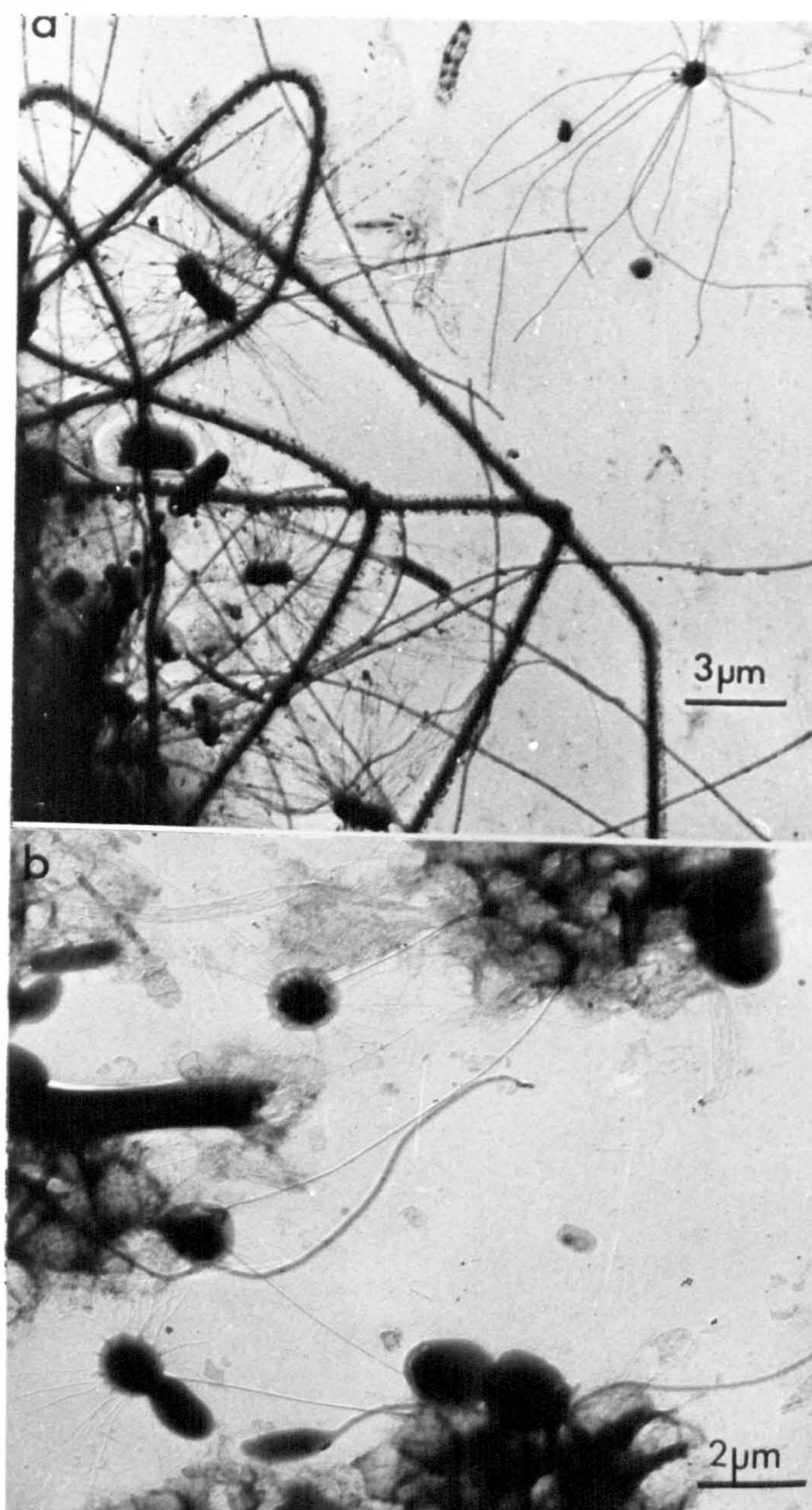


Fig. 2.21

- (a) Multiappendaged bacterium in association with extensively fimbriated cells, within a cellular network.
- (b) Planctomyces and Caulobacter, mutually benefiting from cellular interaction, within the cell complex, both utilising their holdfast to adhere to the substrata, leaving the cell body unrestricted.

(Gold/Palladium shadowed).

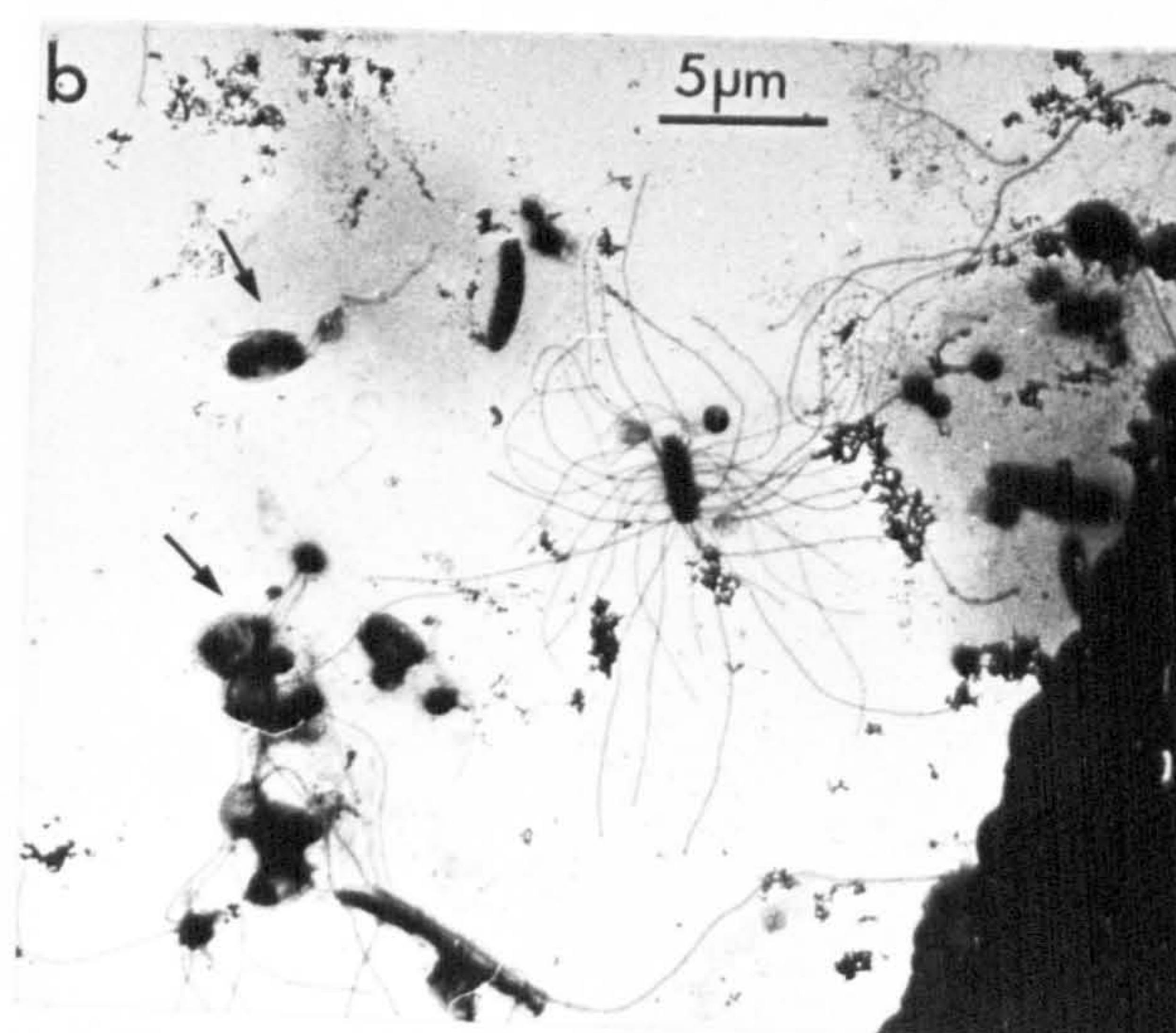
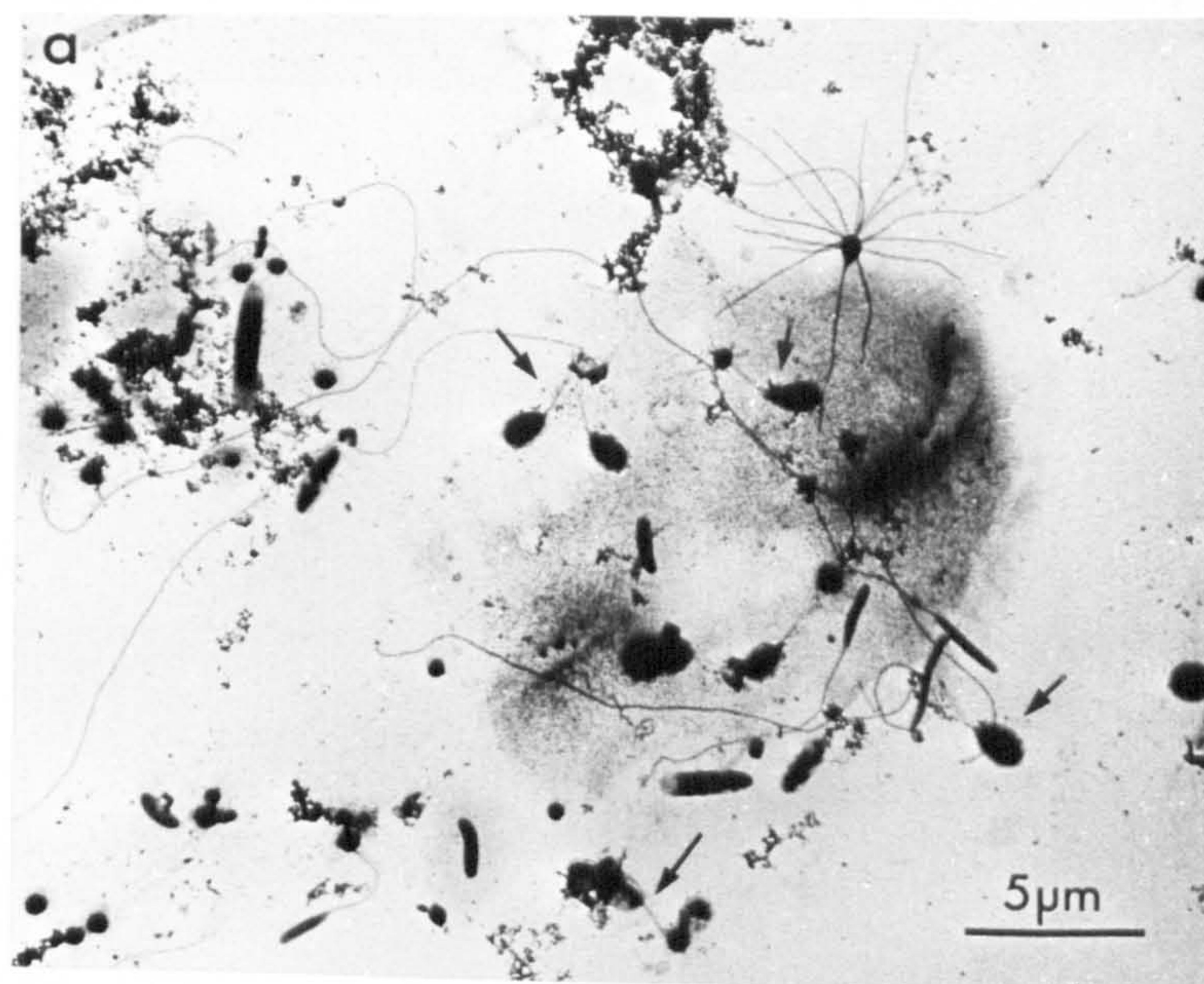


Fig. 2.22

General fields of freshwater enrichments after a few months 'static' incubation, illustrating the adaptive nature of the prosthecate bacteria. Caulobacter and multiappendaged cell stalks are elongated, the stalk of Planctomyces does not appear extended significantly, as the environment becomes devoid of nutrient. (Gold/Palladium shadowed). Arrows indicate Planctomyces.

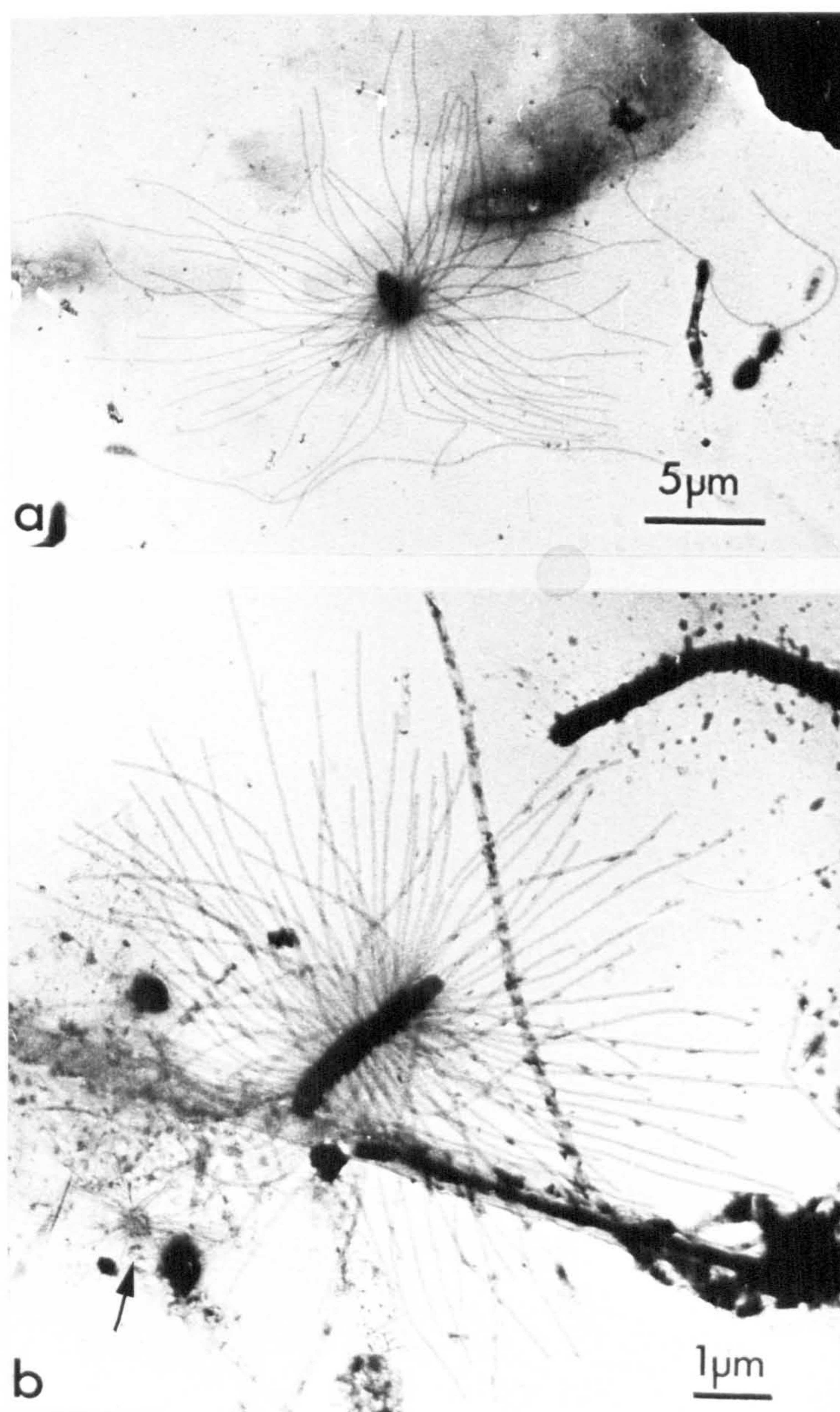


Fig. 2.23 Multiappendaged cells - the phenotype fully expressed in the nutrient poor environment of 'static' incubations. Note presence of fimbriated coccoid cell in (b), in bottom left-hand corner. (12 months incubation).

(Gold/Palladium shadowed).

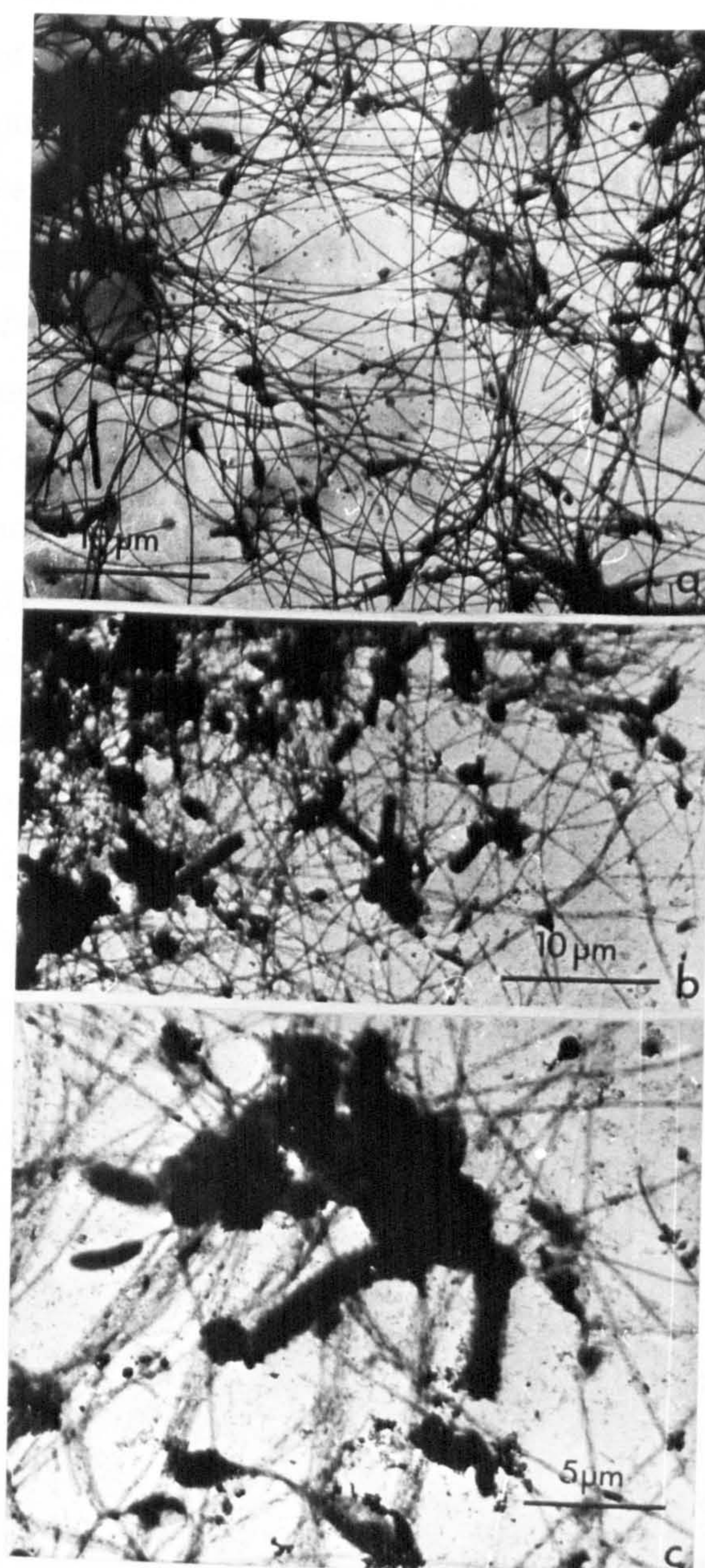


Fig. 2.24

Cellular networks giving rise to a matrix of prosthecae (a).

Rods, when observed, possessed a 'knobbed' structure to their cell surface, and were identified as the nonexpressed phenotypes of multiappendaged rods (b) and (c).

(Gold/Palladium shadowed).

The multiappendaged bacteria enriched for by this slow 'starvation procedure' characteristically possessed very long prosthecae, often in excess of 10 μm (Fig. 2.21a). The cell body was either ovoid or elongated into a rod shape. Unlike Staley's isolates, the number of prosthecae extending from the cell bodies of these bacteria generally ranged from 10 to over 50; Fig. 2.22a shows one of these bacteria observed after 3 months incubation, whereas Fig. 2.22b shows another one observed after 6 months. After 12 months, the number of appendages clearly indicated that they must be performing some essential function towards the survival of the cell in order to justify, energetically, why such structures should be maintained when the cells were essentially starving (Fig. 2.23b). As at no time could these appendages be shown to possess a reproductive function as they do in the case of Hyphomicrobium it would appear likely that these appendages develop in order to increase the surface area for nutrient uptake, as has been previously suggested (Staley, 1968).

Towards the end of the three year study, the water samples had become translucent (Fig. 2.8) and clumps of cells and secreted material were visible in the body of the water. Microscopic examination revealed that these cell clumps were almost exclusively composed of cells with long stalks (Fig. 2.24). Closer examinations indicated that some of these bacteria were Caulobacter or fusiform bacteria (de Bont et al., 1970) (Fig. 2.25), whilst others resembled the generic description of Pedomicrobium (Hirsch, 1974) (Fig. 2.24a). However, detailed studies on pleomorphism in Hyphomicrobium (Section 3.III.4) indicated that Pedomicrobium was in fact a phenotypic variant of Hyphomicrobium, and thus it would appear that these static enrichments had enriched for Hyphomicrobium sp. which were capable of forming multicellular complexes (Fig. 2.24).

Scanning electron microscope studies (S.E.M.) supported the transmission electron microscope (T.E.M.) studies, in showing that these matrix formations had "naturally" developed in this system, rather than being some product of sample preparation (Klainer and

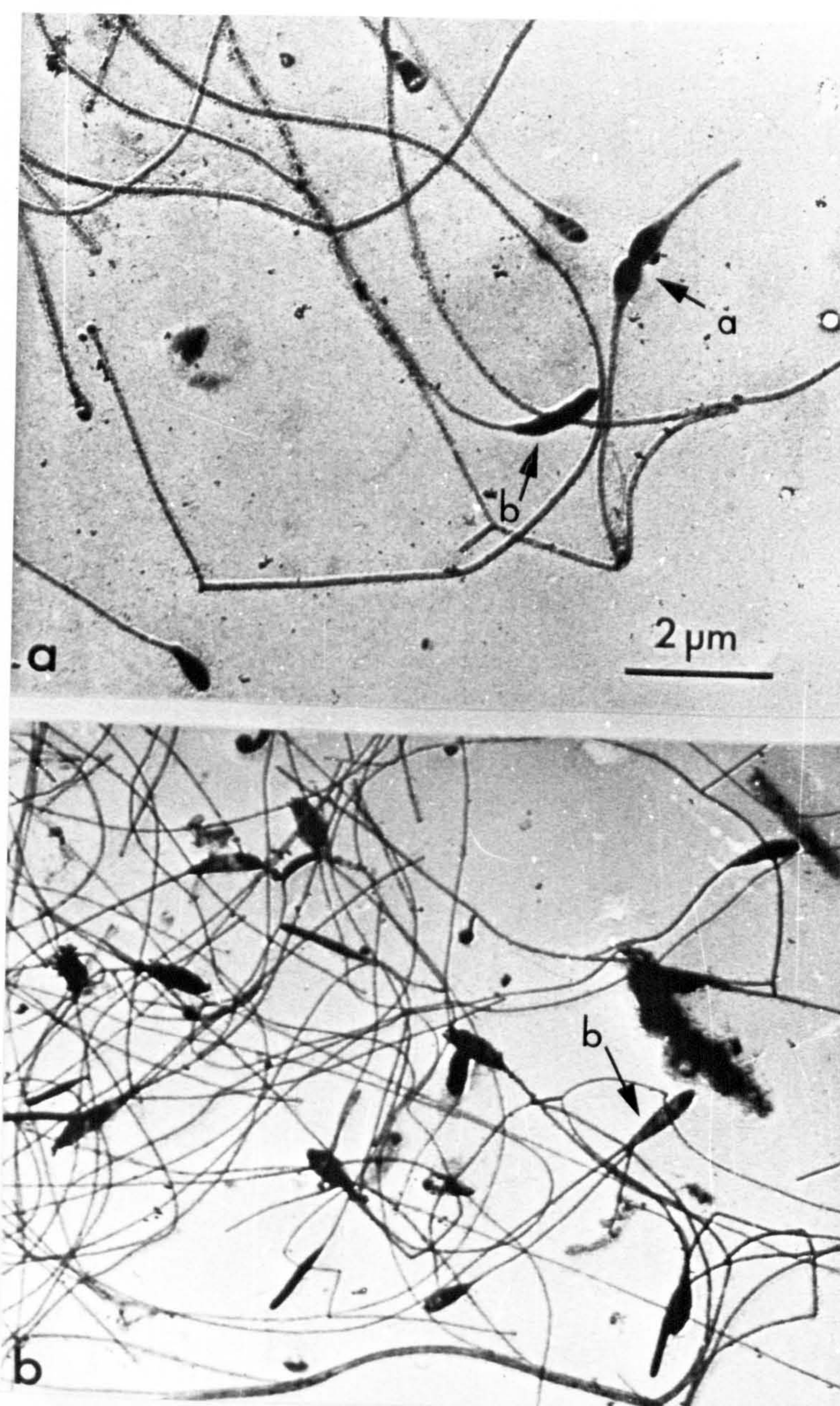


Fig. 2.25

Presumptive cell types responsible for cellular matrix formation included Caulobacter, Prosthecobacter (a) and Hyphomicrobium (b) (see Text). Stalk length was extensive for all three genera, branched prosthecae were attributed to Hyphomicrobium (b). (Gold/Palladium shadowed).

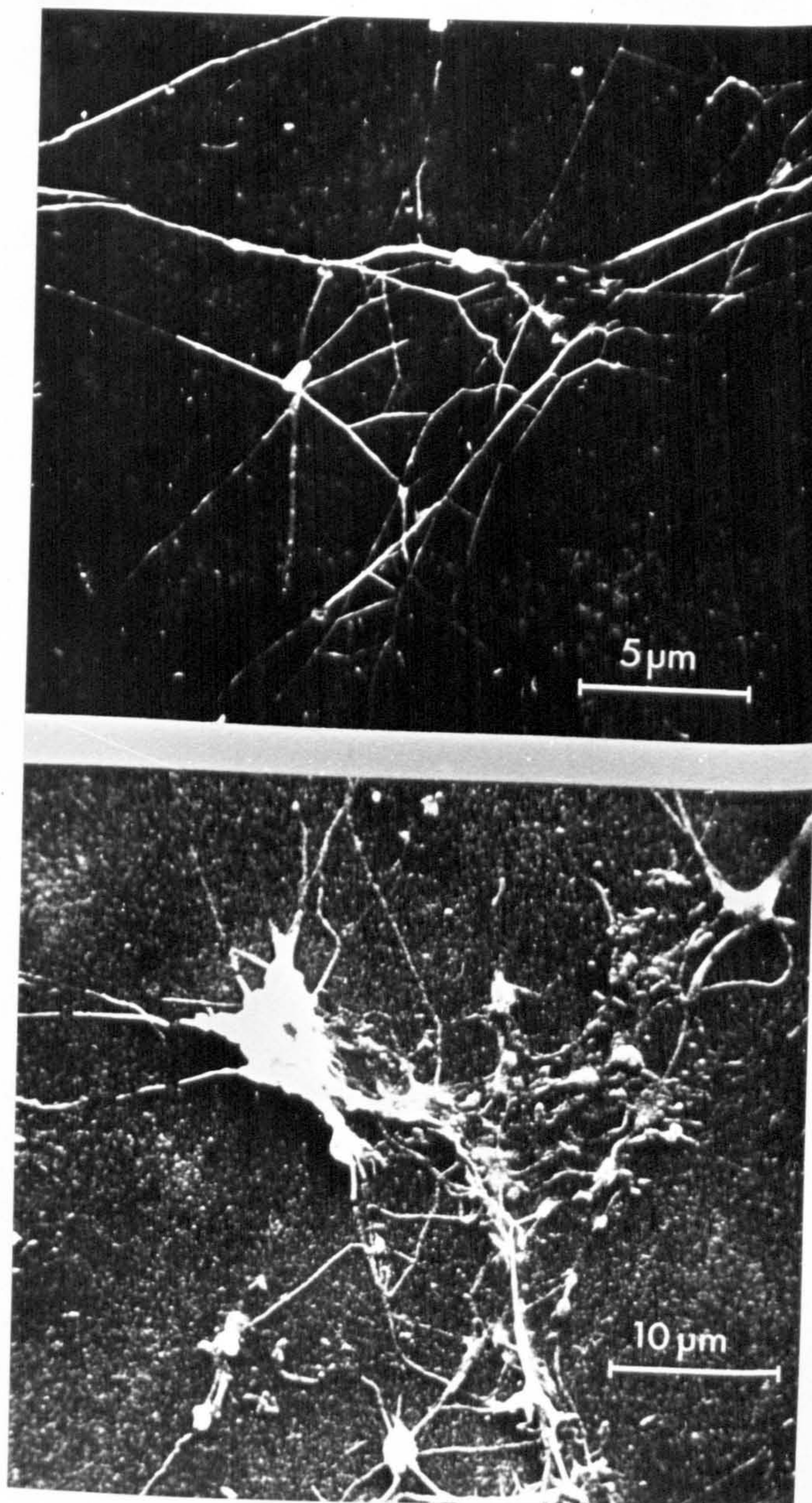


Fig. 2.26

Scanning electron micrographs of freshwater samples after 3 months 'static' incubation. Note extensive prosthecal arrays forming with cross-linking and branching. (Matrix developed on nucleopore membranes).

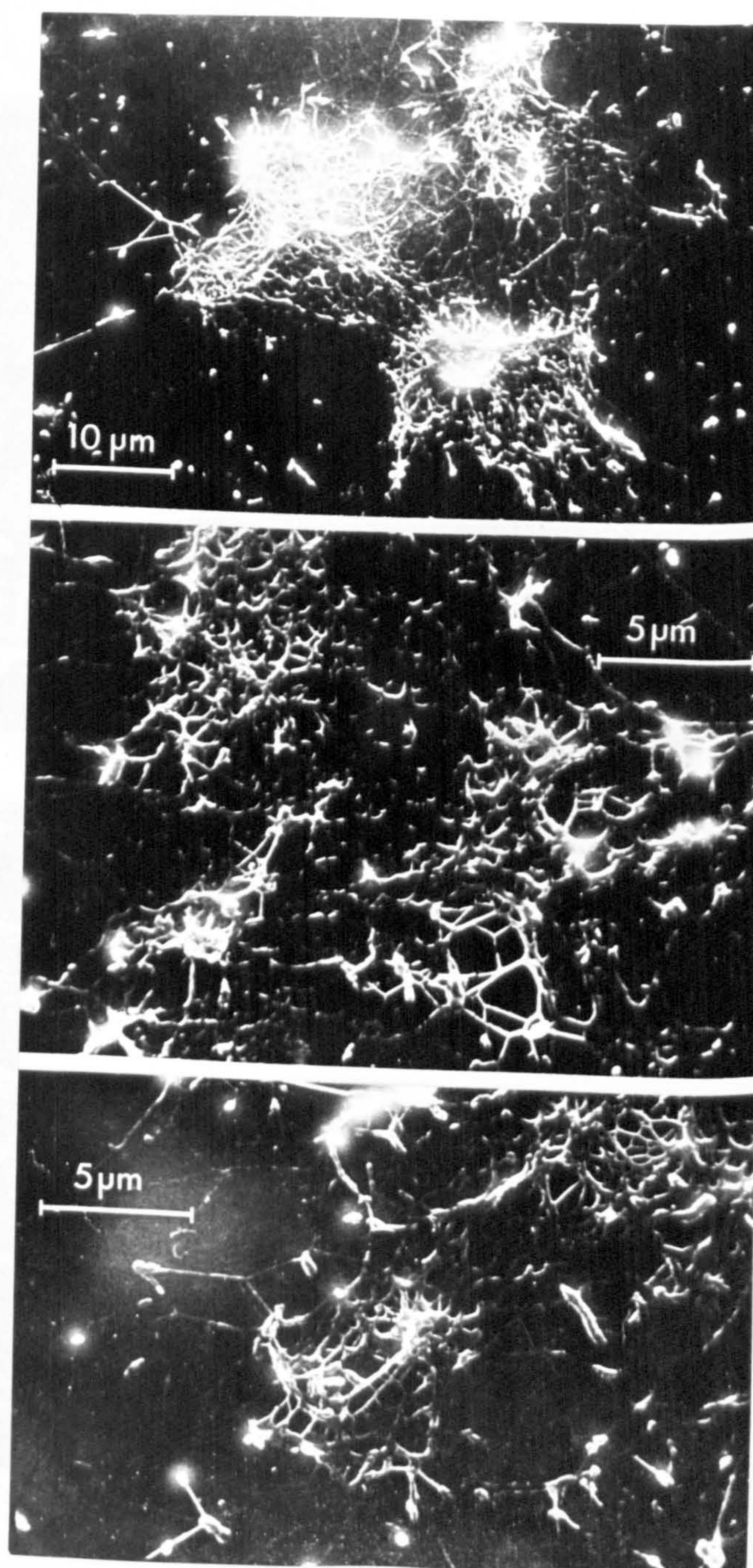


Fig. 2.27

Scanning electron micrographs of water samples. As the arrays aged, the cellular extensions became encased in mucilage, to form solid cell networks [(a) and (b)]. Individual cell bodies emanated from the complex, from prosthecal outgrowths, with the potential to break free, if the situation should so 'dictate' (c). (Nucleopore membrane supports).

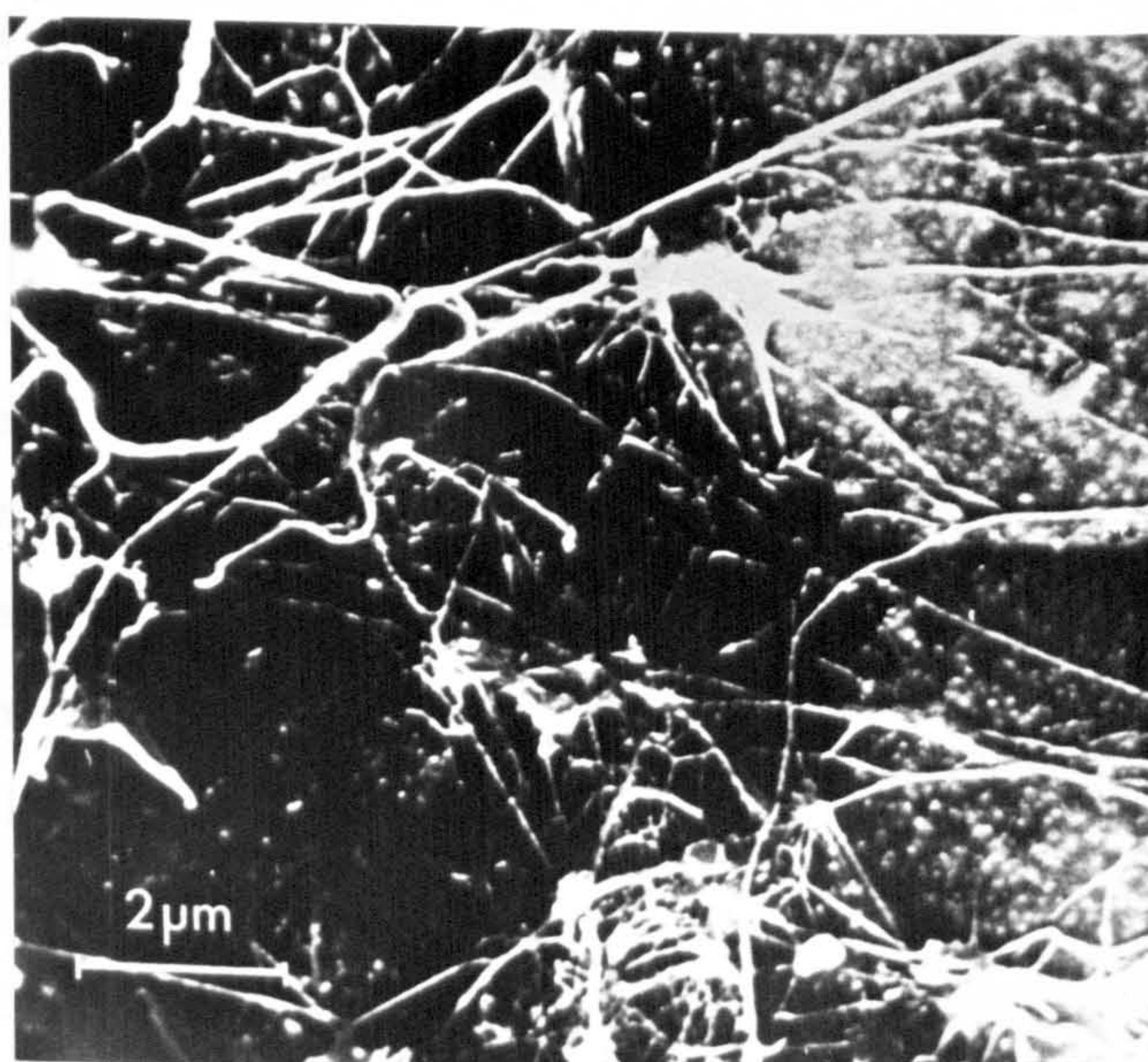
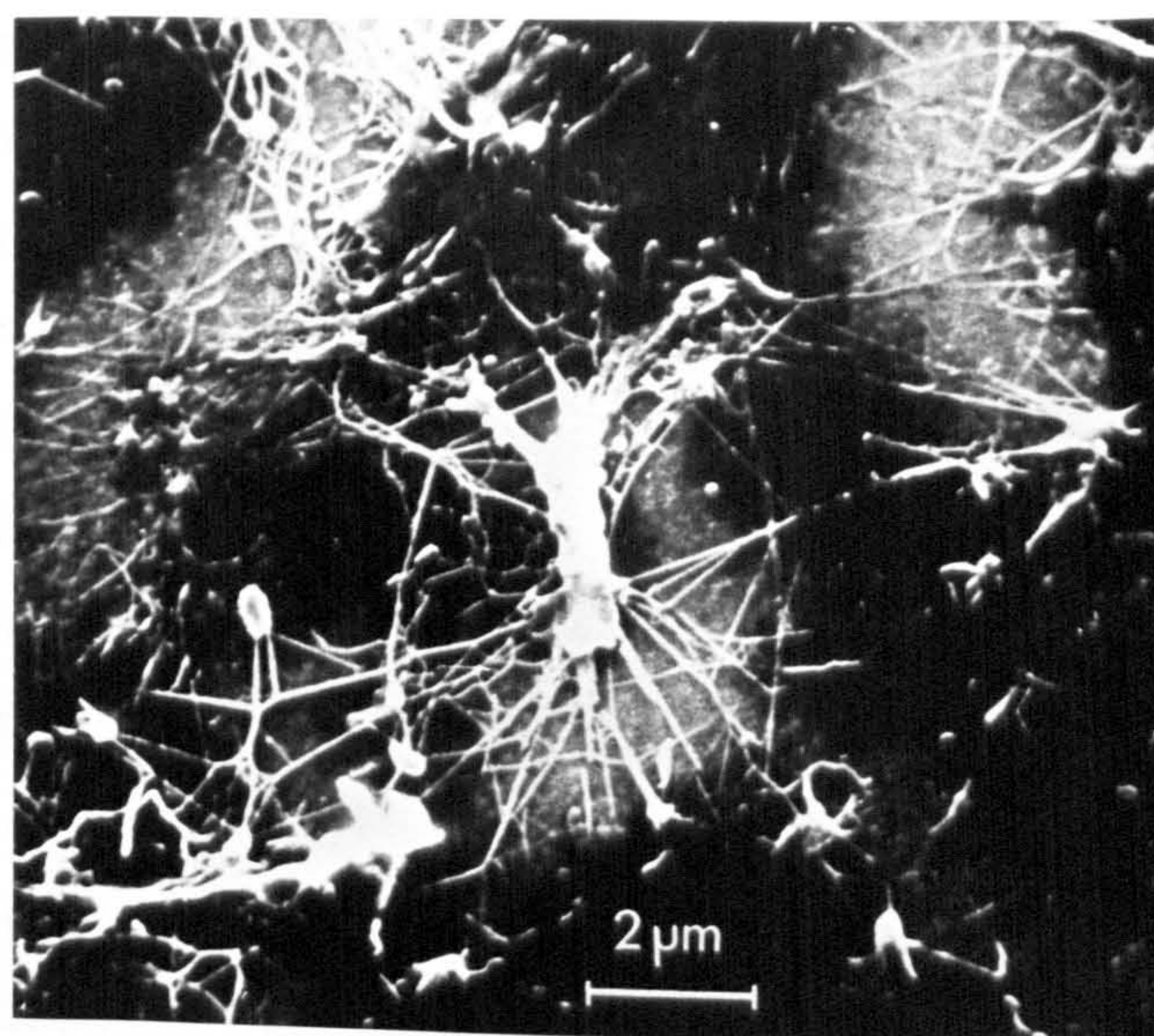


Fig. 2.28

Scanning electron micrography. Expressed phenotypes of multi-appendaged cells within cellular networks, covered with mucilagenous secretions after 1 year's 'static' incubation. Hyphomicrobium cell bodies are apparent. (Support was again nucleopore membranes).

Betsch, 1970)(Figs. 2.26, 2.27, 2.28). Nevertheless, one must bear in mind that such a static system would not develop in the aquatic environment, and so in that respect these matrix formations are artificial. They do show, however, the potential of bacteria to adapt to their environment, although whether this adaptation is a direct or indirect response is not known, and clearly indicate that the prosthecae bacteria are capable of extensive phenotypic variation, which enables them to survive extreme environmental conditions. Hyphomicrobium, known for its ability to exist in very nutrient poor environments, (Tyler and Marshall, 1967a, b; Hirsch and Rheinheimer, 1968), clearly stands out as a microorganism which can successfully adapt to these conditions by morphological variation, and the use of elongated prosthecae as organelles of nutrient uptake.

The effect of C_1 compounds on static enrichments

In order to develop such matrix networks of cells, the system had to be starved of essential nutrients. Consequently routine sampling needed to be carried out rapidly to avoid excessive gaseous exchange above the body of the water. Although the flasks were only stoppered with a thick wad of cotton wool and then covered with foil, whilst the flasks were maintained, the gaseous exchange that occurred was minimal. This proved to be important as the laboratory atmosphere contained methane and ethylene, at levels higher than those found in other laboratories (10 to 30 parts per million compared to 3 parts per million). Repeated sampling from a flask, however, appeared to cause the matrix development to halt, and would particularly cause the multiappendaged bacteria to revert to their non-expressed form (Section 2. III. 2), possibly because the rate of gas exchange increased, and low levels of methane, even with its poor solubility, could partially dissolve in the water (Zavarzin, 1961).

Low levels of C_1 compounds, e.g. methane, methanol, methylamine, formate, formaldehyde, carbon monoxide and carbon dioxide, were added individually to a series of flasks containing water enrichments, throughout

the period of study, at six month intervals, at final concentrations of 1 nmole per litre. The addition of nutrients caused an enhancement of bacterial growth, although stimulation to growth was poor in the cases of carbon monoxide and carbon dioxide. Growth was observed as a slight turbidity in the culture counted under the microscope to correspond to approximately 1×10^6 to 5×10^6 cells per ml.

When these carbon compounds were added initially, they suppressed the development of prosthecate bacteria in the population, whereas if they were added when the prosthecate bacteria were well established, the prosthecae were maintained but the cell matrices disappeared and the multiappendaged cells reverted to a non-expressed form. This reversion of cell phenotype was due to the cell population no longer being under a condition of 'starvation', clearly demonstrating their ability to rapidly adapt to the environment.

2. Budding, prosthecate bacteria from oligotrophic environments

(a) Caulobacter, Asticcacaulis and the fusiform caulobacters

Introduction

Bacteria with a slender extension from one pole of the cell were first described by Jones (1905) and later given the generic name Caulobacter by Henrici and Johnson (1935). When studying micro-organisms attached to submerged slides, two morphologically distinct types were observed; one was vibroid to rod-shaped, which upon division produced a stalked cell and a motile non-stalked cell, and the other was referred to by them as a 'fusiform' type because of the shape of the organism (Bowers et al., 1954). The extensions at that time were considered to be excreted, however Houwink (1955) showed by means of electron microscopy that these stalks were integral cellular extensions and that the cells attached to detritus by means of a localised secretion, the holdfast, situated at the tip of the stalk. Integral cellular appendages, such as the stalk of Caulobacter, are termed prosthecae (Staley, 1968).

In 1962, Stove and Stanier investigated the life cycle of Caulobacter (Fig. 1.8) and showed that prosthecate mother cells gave rise to polarly flagellated, non-prosthecate daughter cells, called swimmers. These eventually shed their flagella and developed a prostheca at the site on the cell surface where the flagellum was inserted (Shapiro and Maizel, 1973). Once the daughter cell has differentiated to produce its prostheca by this obligate temporal sequence of events, it is then and only then capable of producing daughter cells (Fig. 1.8).

Poindexter (1964) also established a new genus, Asticcacaulis, characterised by a subpolar appendage (Fig. 1.14). One unusual species, A. biprosthecum, (Fig. 1.15), typically has two lateral appendages (Pate et al., 1973). Poindexter also observed fusiform strains of Caulobacter and characterised them as C. fusiformis. These strains were motile, whereas those originally observed by Henrici and Johnson (1935) were nonmotile. Staley, de Bont and Jonge (1976)

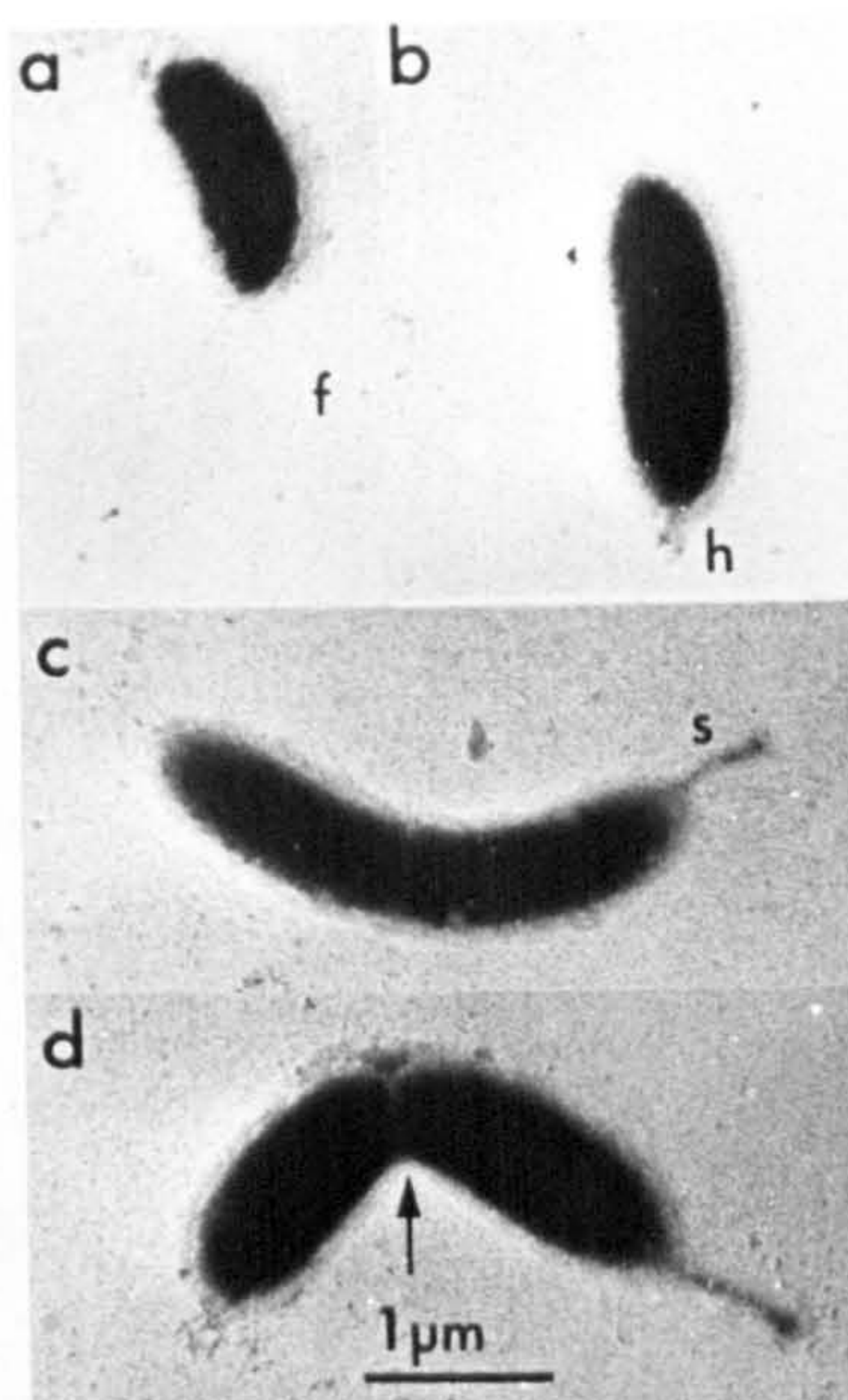


Fig. 2.29 Electron-micrograph of the life cycle of a Caulobacter sp. (b) isolated from Draycote Reservoir. Gold/Palladium shadowed. (f = flagellum, h = holdfast, s = stalk, arrow = plane of division).



Fig. 2.30 Caulobacter sp. from 'static' enrichment system maintained for three years. Crossbands (some are arrowed) appear randomly along the length of the stalk. PTA negatively stained.

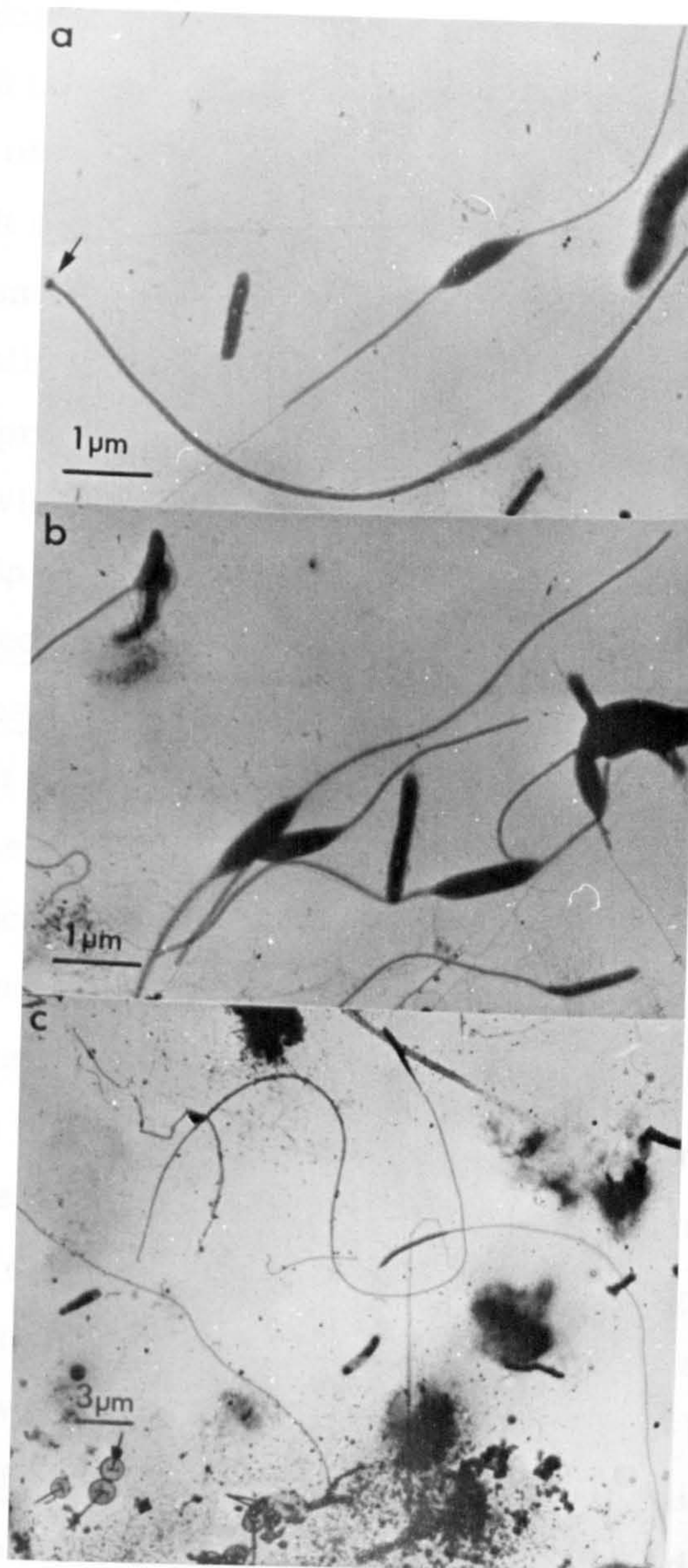


Fig. 2.31 Caulobacter in the natural environment.

- (a) Fusiform bacteria, two types, one bearing bulbous tip (arrowed).
 - (b) Mixed group of Caulobacter and fusiform bacteria.
 - (c) General field from Draycote Reservoir of caulobacters.
(Umbrella-shapes also present - arrowed).
- (Gold/Palladium shadowed).

isolated four strains of nonmotile fusiform bacteria from freshwater sources, supporting earlier studies by de Bont, Staley and Pankratz (1970) which agreed with the description of Henrici and Johnson (1935) of a single prostheca extending from one pole of mature cells, and cells attached to various substrata by means of a holdfast located at the distal tip of the appendage. Unlike Caulobacter, these bacteria did not exhibit a dimorphic life cycle, as both mother and daughter cells were immotile, and at the time of separation the daughter cells were essentially mirror images of the mother cells (Fig. 1.16). In addition, the prosthecae of the fusiform bacteria did not show cross-bands, were wider than the stalks of Caulobacter sp. and terminated in a bulbous tip (Fig. 2.31a). Staley et al. (1976) proposed a new genus, Prosthecobacter fusiformis for these fusiform caulobacters.

Characteristics of the stalk in Caulobacter and Asticcacaulis

The wall of the stalk is continuous with both the lipopolysaccharide and mucopolypeptide layers of the cell wall (Haaers and Schmidt, 1974). The only distinctive structure found in these appendages are the cross-bands (Jones and Schmidt, 1973). However, recent studies (Retnasabapathy, personal communication) have shown that, although the number of cross-bands in stalks does increase through successive generations, there does not appear to be any direct correlation between the number of crossbands and the number of generations an individual cell has gone through. Furthermore, the distance between the crossbands is not constant, and appears to reflect the growth rate of the cell, i.e. the distance is greater at slow growth rates (cf. Staley and Jordan, 1973). Fine structure studies in Caulobacter (Poindexter and Cohen-Bazire, 1964) and Asticcacaulis (Pate and Ordal, 1965) have shown these crossbands to be composed of solid annular structures of mucopolypeptide.

Function of the stalk in the natural environment

A consideration of the structural similarities of the stalks of Caulobacter and the 'pseudostalks' of Asticcacaulis has led to these being considered as homogenous structures (Pate and Ordal, 1965). The property of attachment has been attributed to the stalk of Caulobacter (Houwink, 1952); however, this is in fact a property of

the secreted holdfast, and can occur in the absence of the stalk (Poindexter and Cohen-Bazire, 1964). The possible function of these prosthecae as a means of increasing surface area for nutrient uptake, thereby giving these organisms a selective advantage for survival in dilute nutrient environments, has already been indicated (Section 2.III.1) (Fig. 2.30). Schmidt and Stanier (1966) observed a marked elongation in stalks by the limitation of phosphate; however, Schmidt (1971) showed that stalked Caulobacter cells fail to take up nutrients more efficiently than swarmer cells. Later studies (Degnen and Newton, 1972a, b; Newton, 1972; Schmidt and Samuelson, 1972) indicated that variations in stalk length was a direct consequence of the growth rate which was influenced by the available phosphate, possibly through the action of nucleoside and nucleotide phosphate pools within the cell (Kurn et al., 1975). Larson and Pate (1976) have shown active transport of glucose in prosthecae isolated from cells of Asticcacaulis biprosthecum, and cells which lack these cellular extensions have now been isolated (Pate et al., 1973). These mutants appear to be very similar to the wild type with asymmetric division with respect to size, i.e. small swarmer cell and larger mother cell. No stalk or pili are observed, but the flagellum is still present, although inactive. These studies suggest that, as the swarmer cell undergoes transition to cells of a stalk cell nature without stalk formation, and the cell cycle of stalkless mutants proceeds in an ordered sequence similar to that defining the wild type cell cycle (Shapiro, 1976), the stalk formation is not obligate to the life cycle as previously thought (Schmidt and Stanier, 1966; Staley and Jordan, 1973).

Bacteria from the genera Caulobacter and Prosthecobacter were frequently observed in freshwater samples and enrichment cultures (Fig. 2.22). Attempts were made to isolate and study this group of bacteria in order to ascertain the function of the stalk in nutrient poor environments.

Results and Discussion

Freshwater isolates of *Caulobacter* and *Prosthecobacter*

Cultural characteristics and physiology

Several isolates of *Caulobacter* were obtained using the peptone enrichment of Staley and Mandel (1973) (Section 2. II.3) and characterised using the descriptions of Poindexter, (Bergey's Manual of Determinative Bacteriology, 8th Edn., 1974). These are described below:-

- (a) Colonies, pale yellow, mucoid, reaching a maximum diameter of 4 mm.

Cells, vibroid, with a short stalk.

Yeast extract essential for growth.

- (b) Colonies, bright yellow, firm, maximum diameter 2 mm.

Cells, slightly vibroid, distinctive stalk.

Vitamin B₁₂ needed for growth; yeast extract also stimulated growth.

- (c) Colonies, small and colourless. (Diameter, 2 mm).

Cells, slender vibroid, long stalk.

Yeast extract or vitamin solution needed for growth.

The overall morphology of each organism, as revealed by phase microscopy was confirmed by electron microscope examination.

Although fusiform bacteria were considerably enriched for, none were isolated in pure culture (Fig. 2.31). When liquid cultures of these organisms were incubated on a rotary shaker, the culture fluid became evenly turbid, whereas static cultures developed as a pellicle. The optimum temperature for growth was 30° C, at pH 7.2 in aerated Sg liquid cultures. Cultures grew well in Sg medium with glucose as carbon source, but poorly on defined medium (GMB). Doubling times as determined by spectrophotometric measurement (Section 3. II) were 150 mins and 12 hours respectively for isolate (b), grown on complex and defined media.

One-carbon compounds, substituted for glucose in GMB medium, were not utilised; however, ethanol did give marginal growth over a control with no added carbon source. Studies indicated that isolates (b) and (c) were identical (Figs. 2.29, 2.32) whereas isolate (a) was characterised by long-cell bodies, especially in defined media (Fig. 2.33). Only isolate (c) bore a long prostheca (Fig. 2.34a); it also grew poorly compared to the others (30 hours doubling time in defined media).

(b) Multiappendaged organisms: *Ancalomicrobium* and *Prosthecomicrobium*

Introduction

Morphologically exotic organisms have been observed both by light and electron microscopy in freshwater, mud and soil samples (Henrici and Johnson, 1935; Nemec and Bystrický, 1962; Stefanov and Nikitin, 1965; Orenski, Bystrický and Maramorasch, 1966a, b; Nikitin, Vasily'eva and Lokmacheva, 1966; Nikitin and Vasily'eva, 1967; Nikitin and Kuznetsov, 1967; Hirsch and Rheinheimer, 1968; Staley, 1968; Bystrický 1970; Volarovich and Terent'ev, 1970; Old and Wong, 1972, 1974; Nikitin, 1973). Isolates of the unusual Gram negative organisms, *Ancalomicrobium* and *Prosthecomicrobium* have been cultured and studied in the laboratory (Staley, 1968; Staley and Mandel, 1973; Moore and Staley, 1976; Dow et al., 1976). Both genera are characterised by several cellular extensions per cell. Reproduction in the case of *Ancalomicrobium* appears to be by asymmetric polar growth (i.e. budding (Staley, 1968).

Results and Discussion

Freshwater isolates of multiappendaged organisms

Enrichment cultures were routinely screened for budding, prosthecate or appendaged bacteria. Multiappendaged bacteria were frequently observed attached to detritus (Fig. 2.36), in cell aggregates (Fig. 2.37) or free in the aquatic environment (Figs. 2.38, 2.39). Many different forms were observed, confirming previous

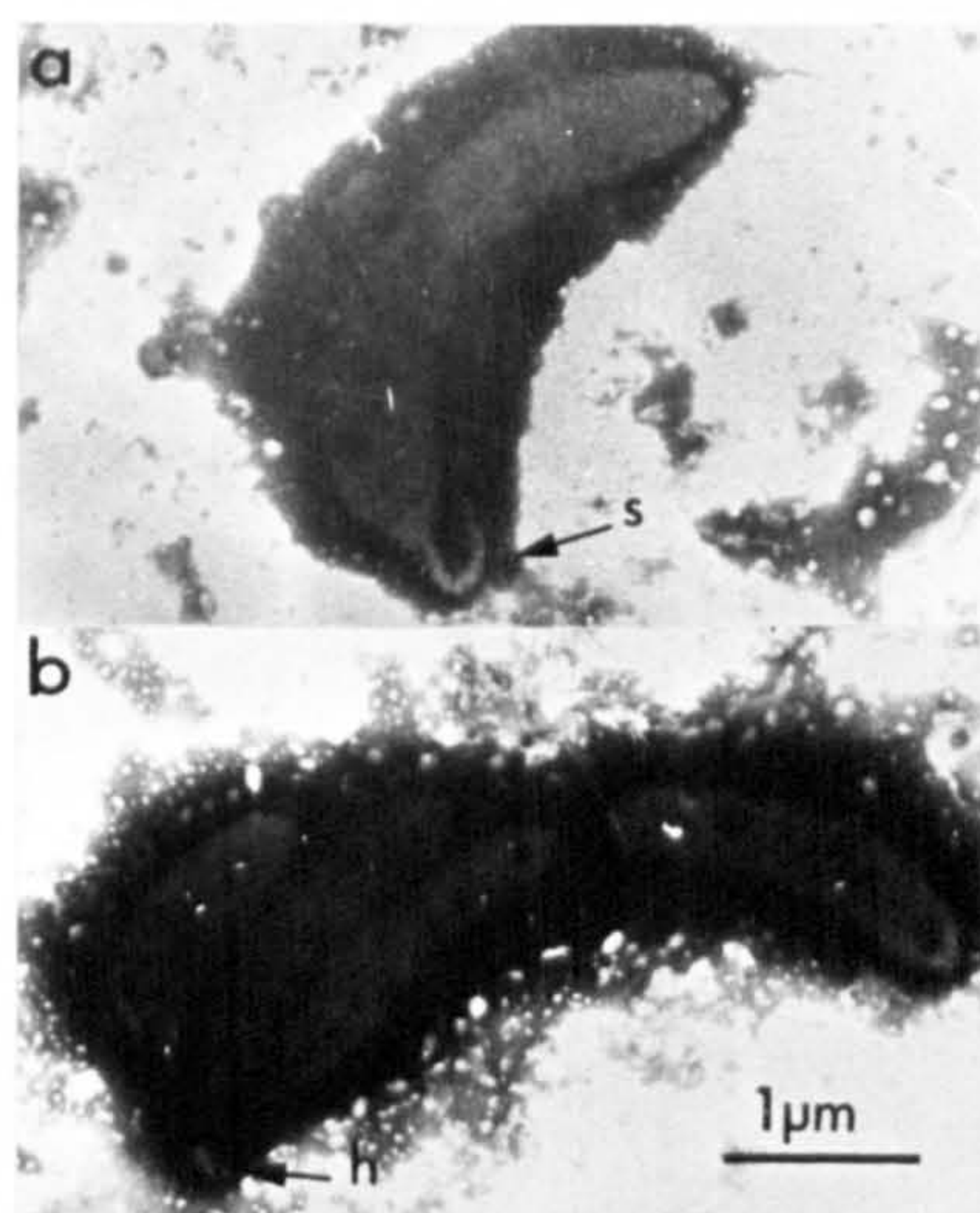


Fig. 2.32 Negative stained preparation of Caulobacter, isolate b showing (a) short stalk and (b) rosette formation. (s = stalk, h = holdfast)

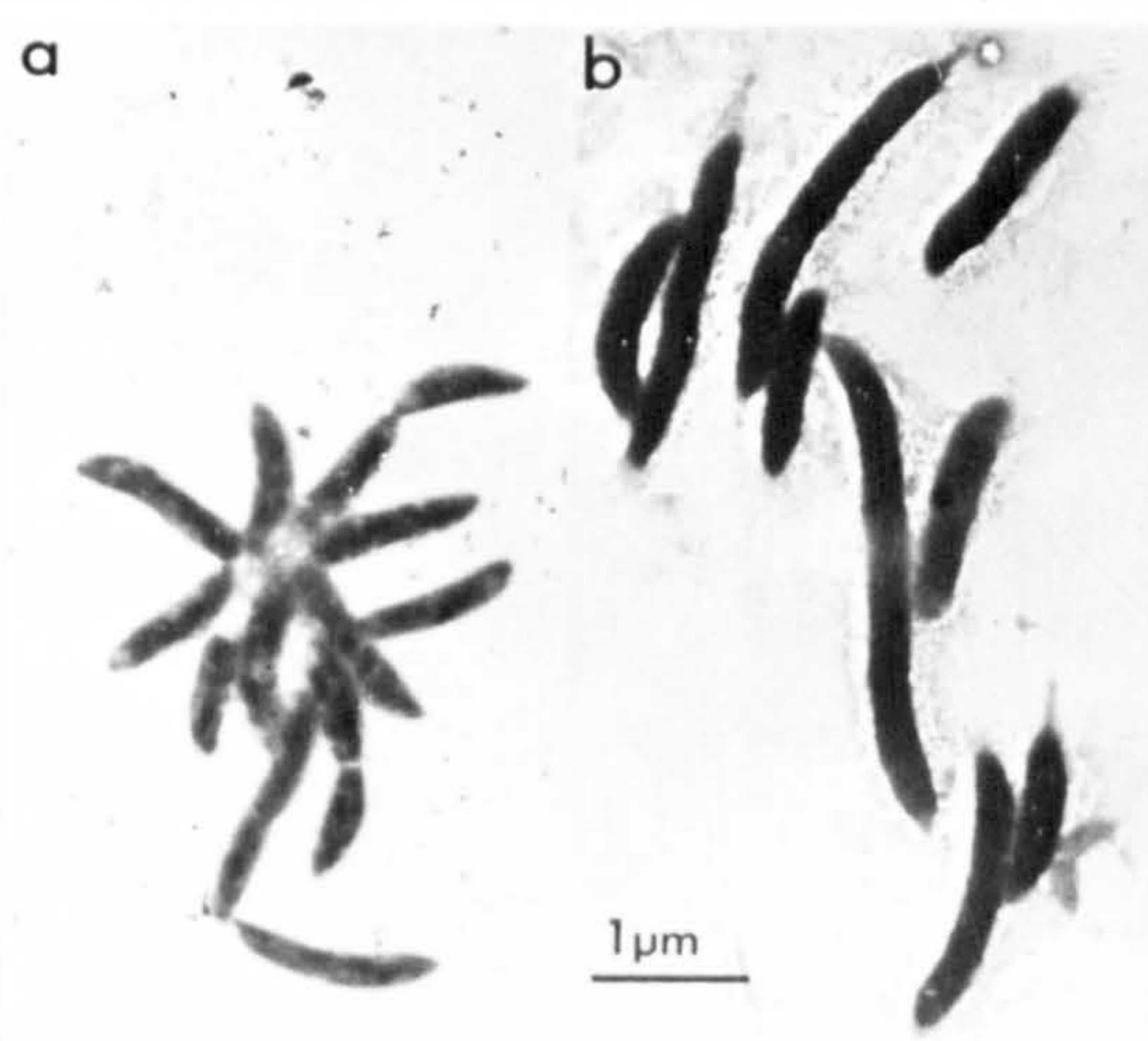


Fig. 2.33 Electron micrographs of Caulobacter, isolate a, illustrating (a) rosette formation, (b) cells in response to growth in defined media (normally grown in complex media) became elongated. (Gold/Palladium shadowed).

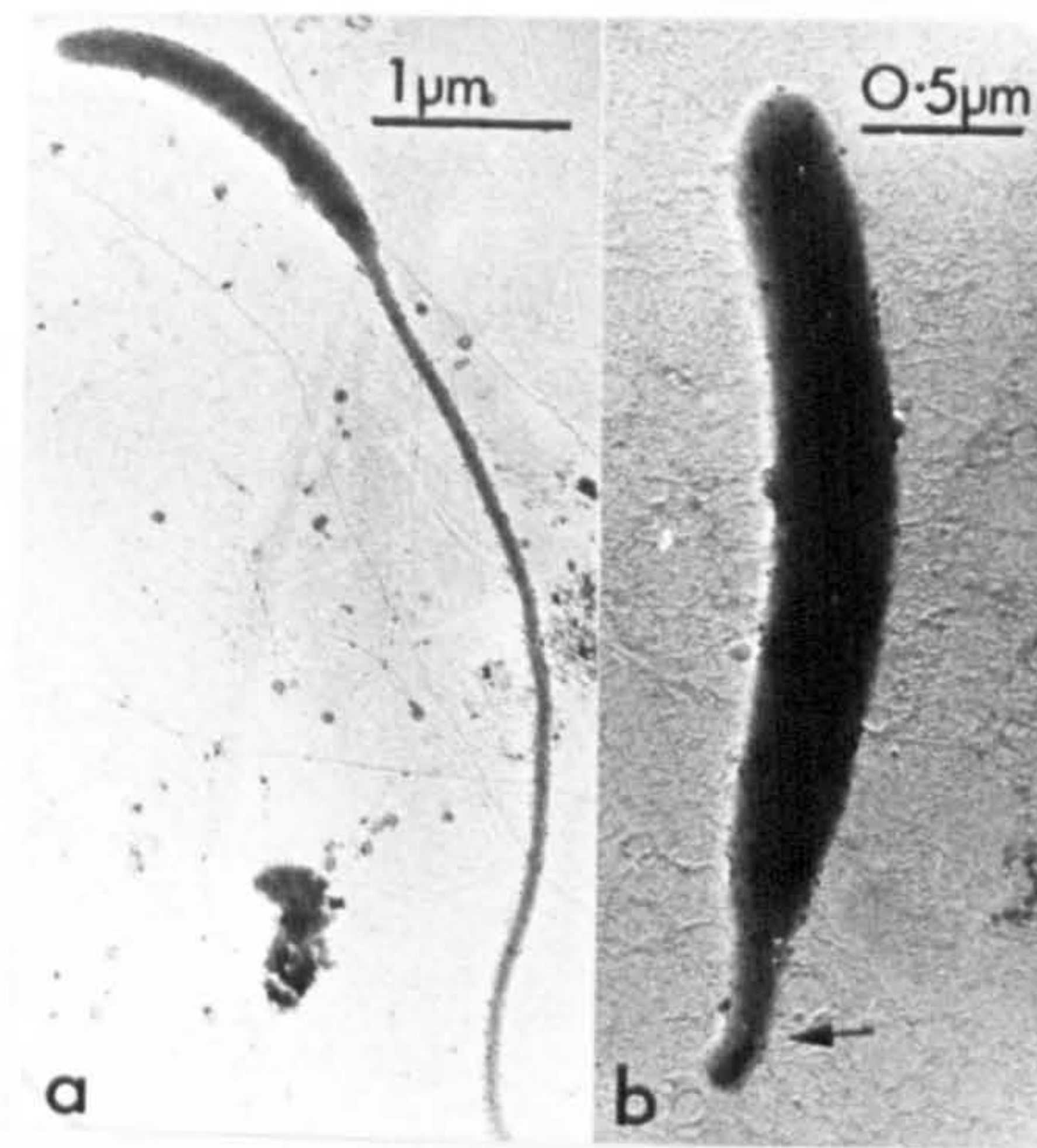


Fig. 3.34 (a) Caulobacter, isolates (c) and (b), Caulobacter, isolate (a), showing the diverse morphology of this genus. (Gold/Palladium shadowed).

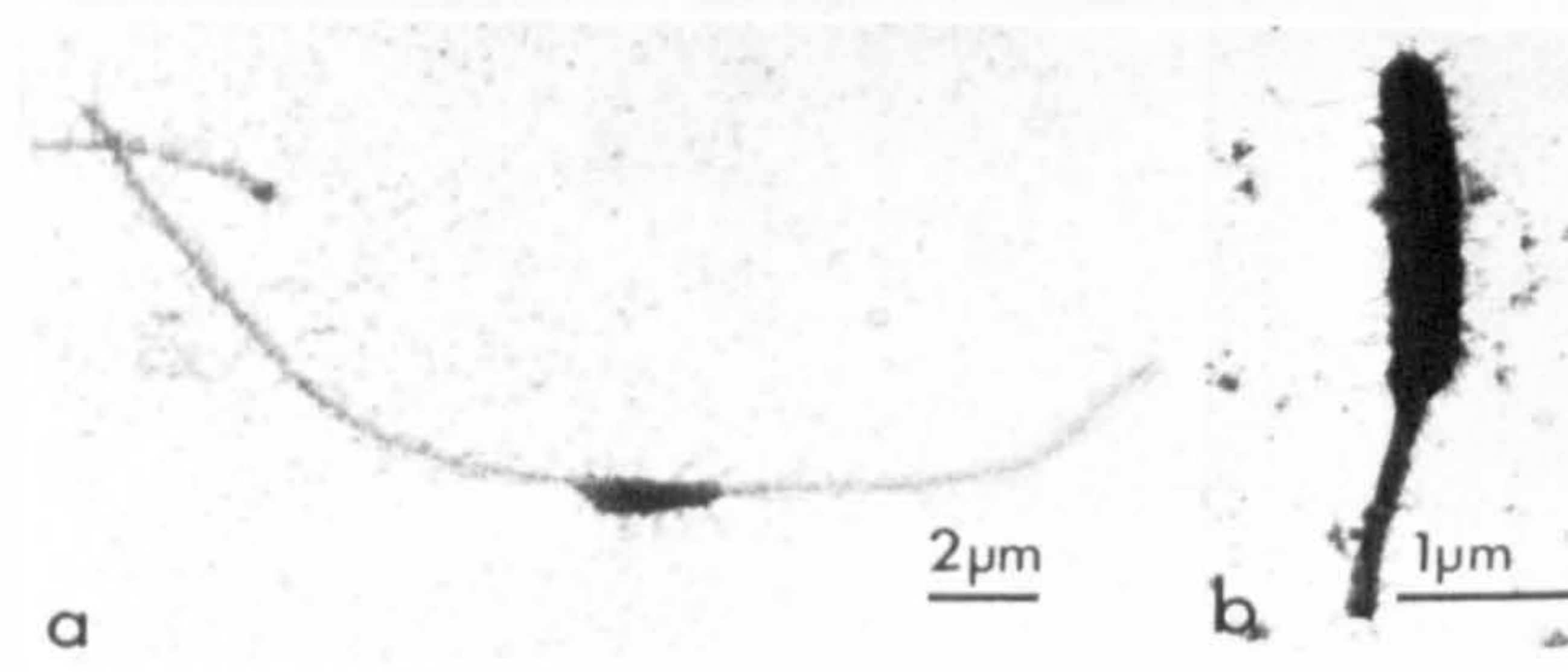


Fig. 2.35 Electron micrographs of a fusiform Caulobacter (a) and Caulobacter sp. (b) from Haweswater, showing arrays of short fimbriae about the surface of cell body and stalk(s). (Gold/Palladium shadowed).

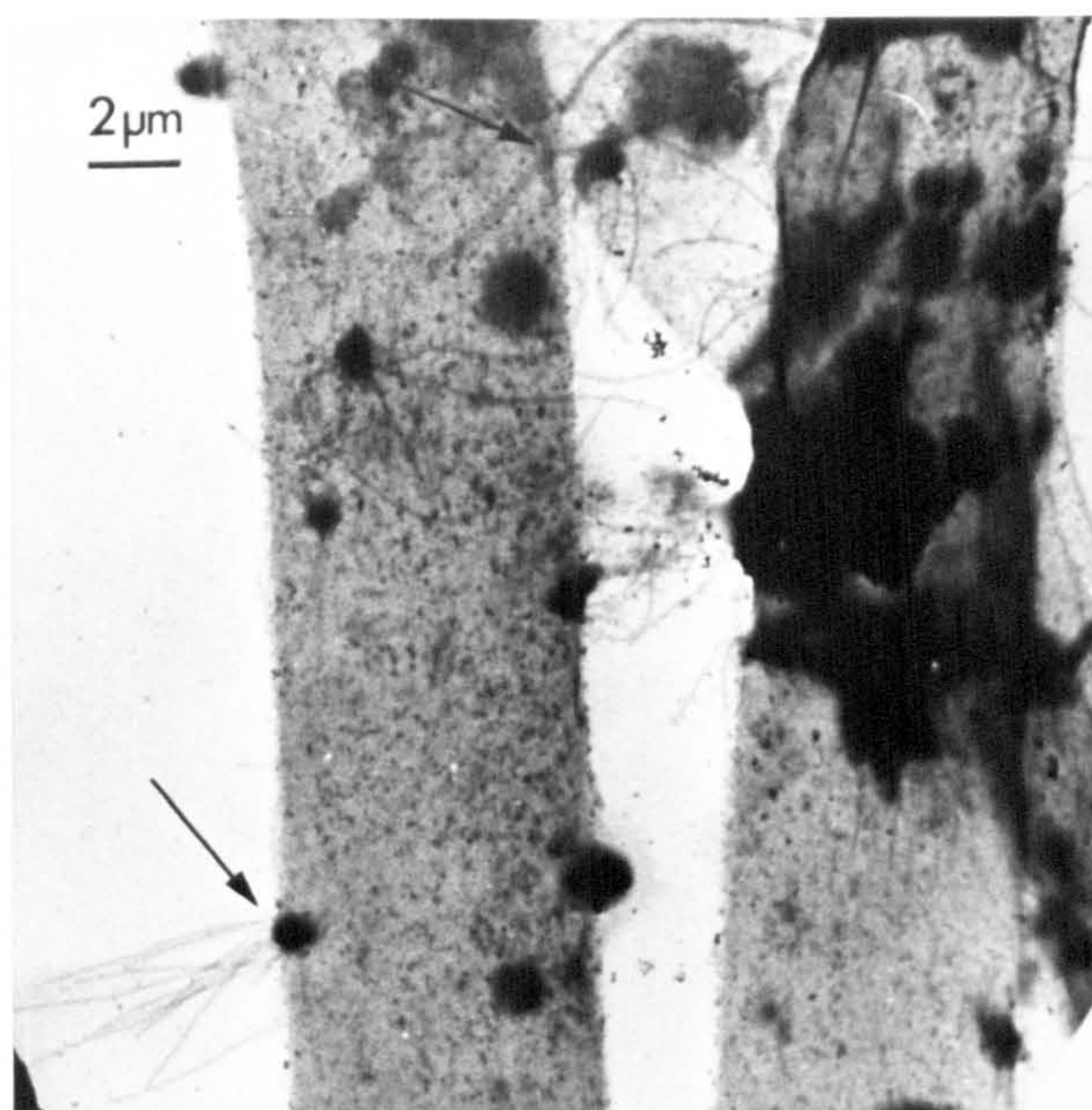


Fig. 2.36 Electron micrograph of multiappendaged cells (arrowed) attached to detritus. (Gold/Palladium shadowed).

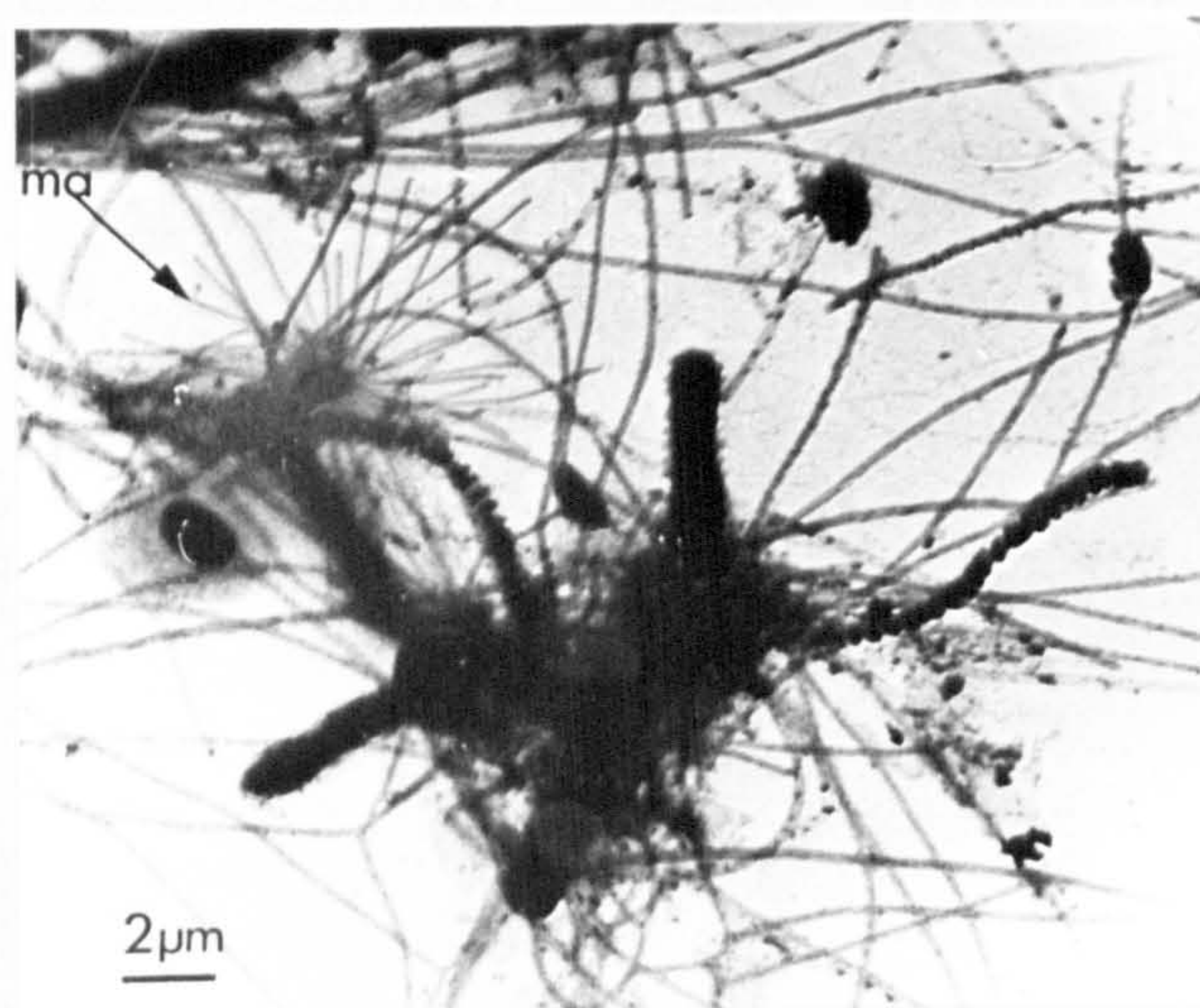


Fig. 2.37 Multiappendaged cells (arrowed) in expressed and non-expressed forms (the knobbled rods), in cell aggregates. (Gold/Palladium shadowed).

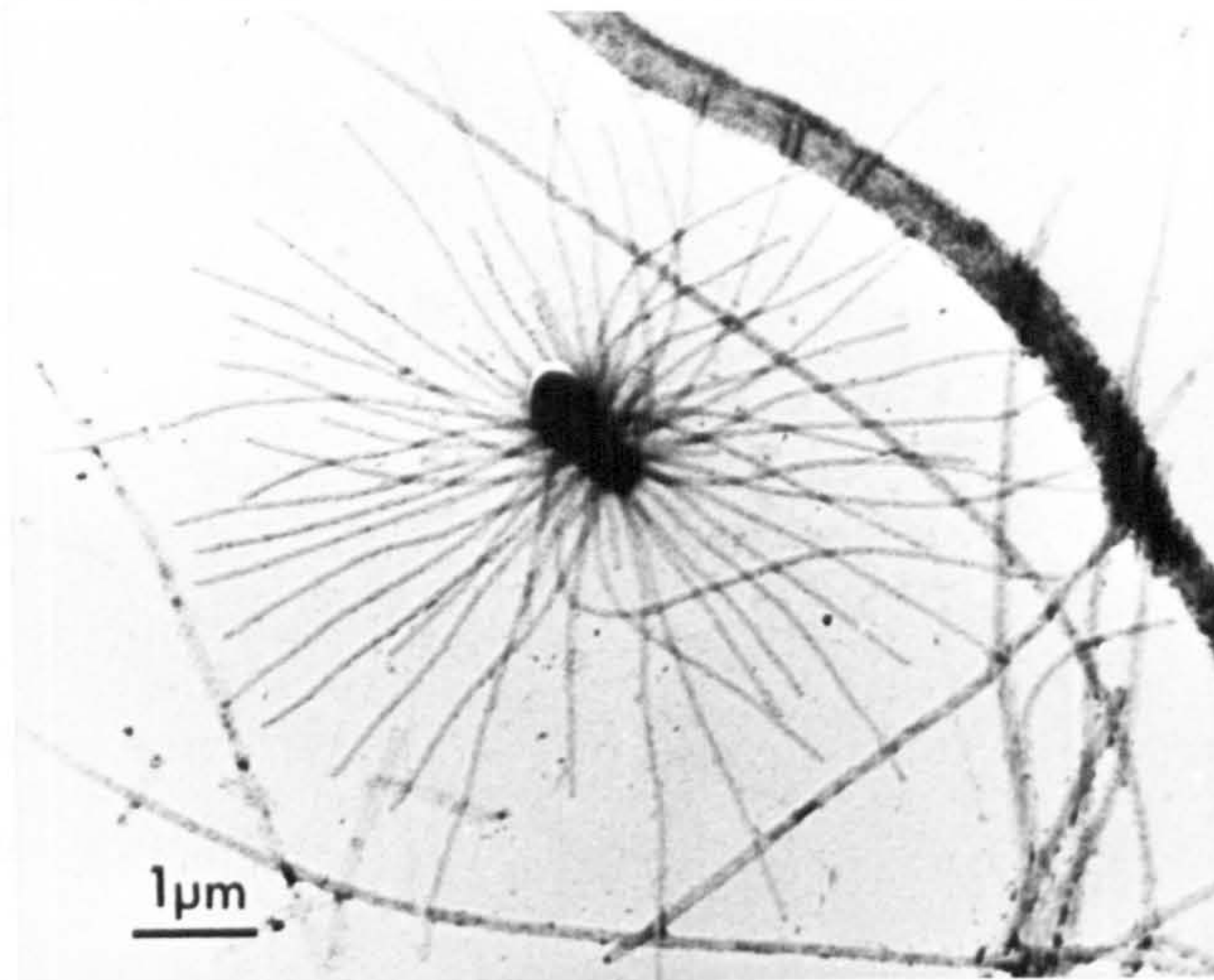


Fig. 2.38 Multiappendaged bacterium, free in the aquatic environment, prosthecae extending from over the whole cell surface. (Gold/Palladium shadowed).

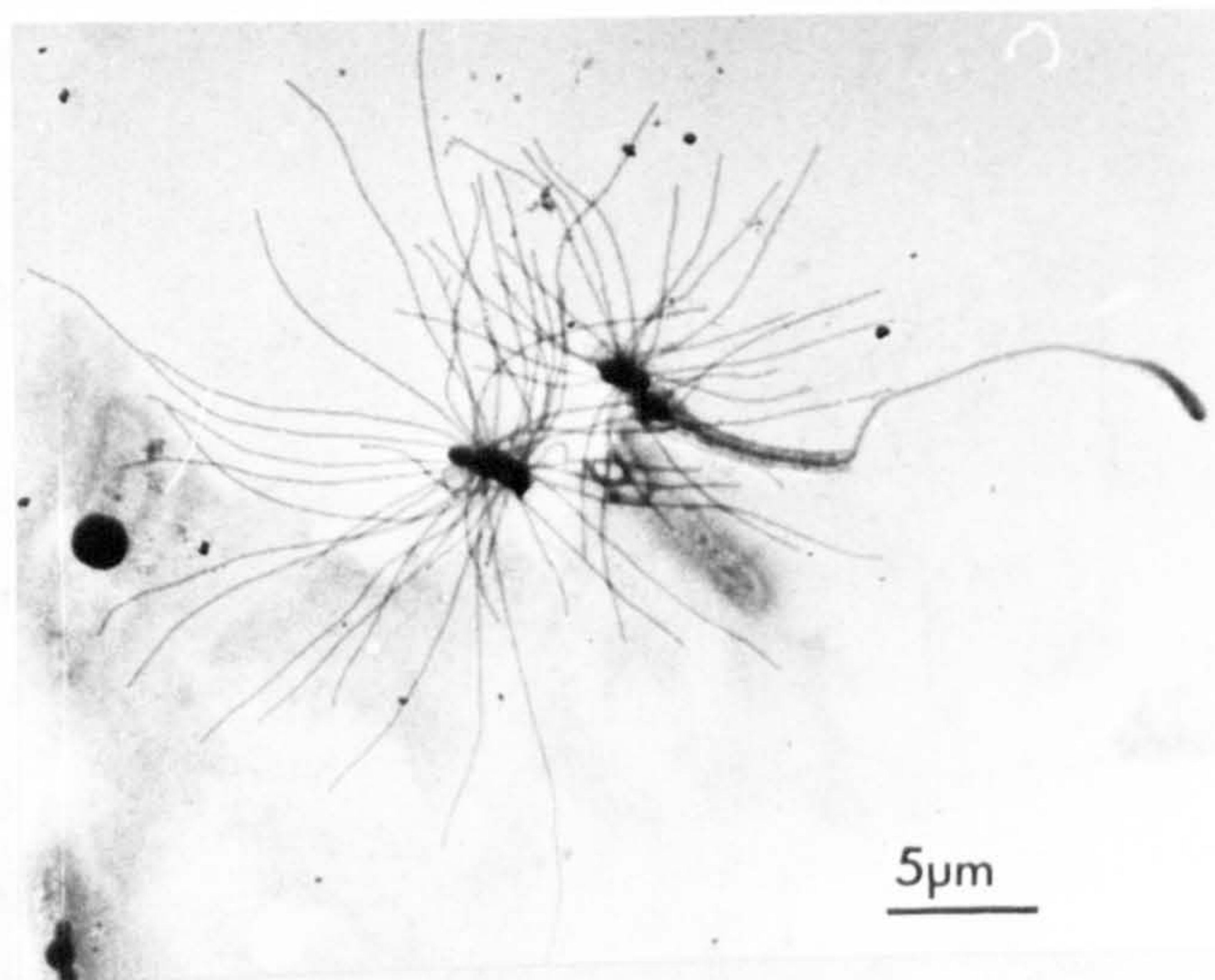


Fig. 2.39 Two multiappendaged cells in association with a *Caulobacter* sp., whose stalk appears to be partly ensheathed. (Gold/Palladium shadowed).

studies (Nikitin et al., 1966; Staley, 1968), however, previously undescribed cell types were also seen in the enrichment cultures, especially the static cultures, with no nutrients added, which appeared capable of extensive morphological variation under different environmental conditions, agreeing with observations made by Dow et al., (1976).

Two multiappendaged organisms were isolated and designated D. 1, 2 and D. 1, 4. Detailed studies were carried out on these isolates and the results were compared with observations made on multi-appendaged bacterial species in the natural environment.

Physiology

Isolate D. 1, 2 on initial isolation appeared identical to Ancalomicrobium adetum (Staley, 1968) in growth and morphology (Figs. 2.40, 2.42). The cells grew aerobically on peptone supplemented with vitamin solution (medium PWP), pH 7.0, at 30° C to give beige, slightly mucoid colonies on solid media, but grew poorly in liquid media.

Isolate D. 1, 4 appeared to resemble Tuberoidobacter (Nikitin et al., 1966) with the cell body having a very granular appearance with protuberances extending from all over the cell surface (Fig. 2.41). Routine maintenance was on PWP medium, at 30° C, giving rise to white colonies. Again there was poor growth in liquid media.

Morphology and morphological variation

Isolate D. 1, 2

These Gram negative multiappendaged cells characteristically possessed from two to eight long and often tapered cellular appendages (prosthecae.) of length approximately 3 μ m, which extended all over the cell surface. The prosthecae occasionally bifurcated, but were never observed bearing buds. Individual cells appeared to be non-motile and to be without a holdfast structure; no vacuoles were observed in the body of the cell.

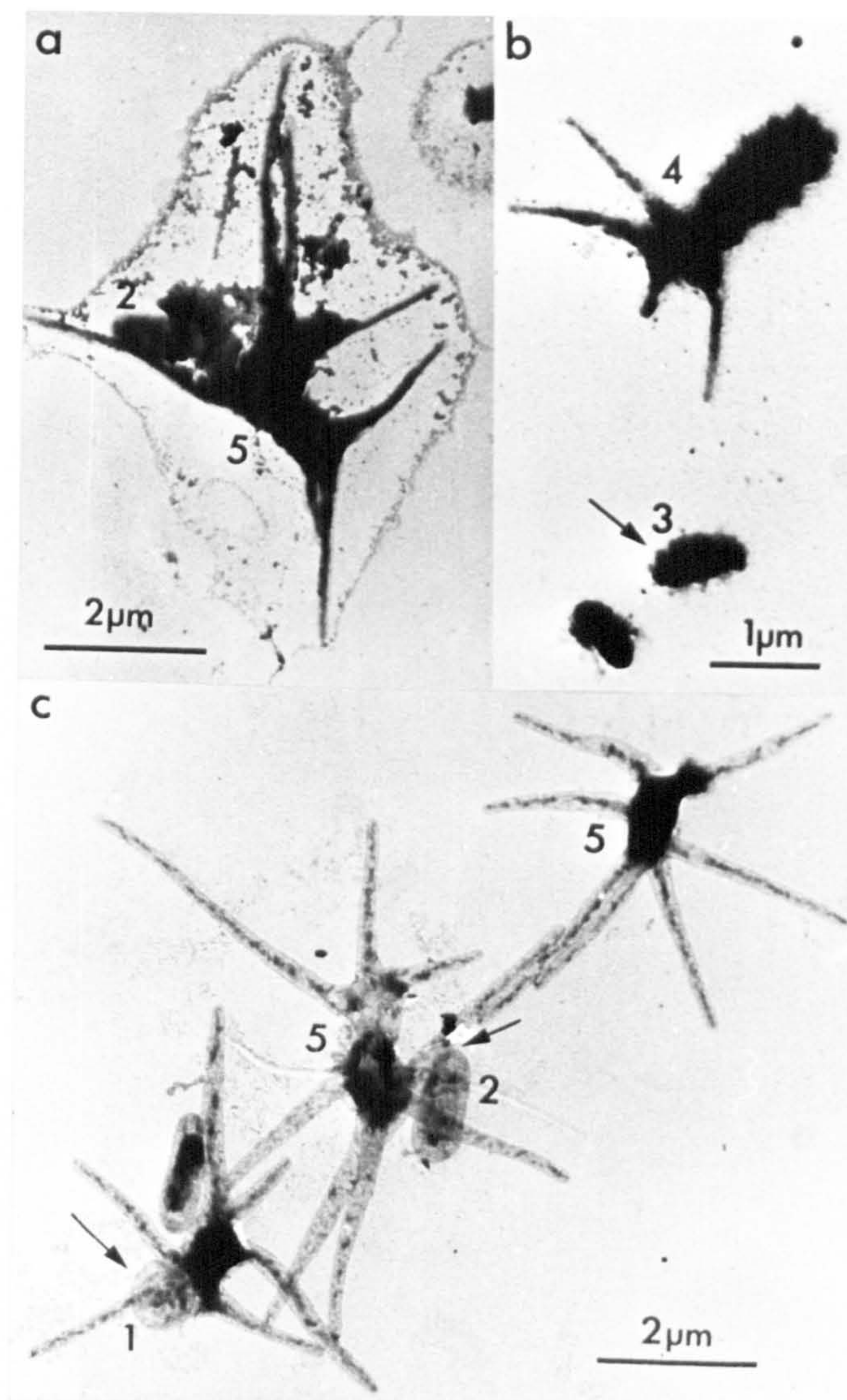


Fig. 2.40 Electron micrographs of isolate D.1,2. Numbers represent various phenotypes observed in a liquid culture, described in Fig. 2.1. (a) Unwashed preparation, cells surrounded by mucilagenous material. (b) and (c) Washed cells illustrating the phenotypic variation expressed by this bacterium. (Gold/Palladium shadowed).

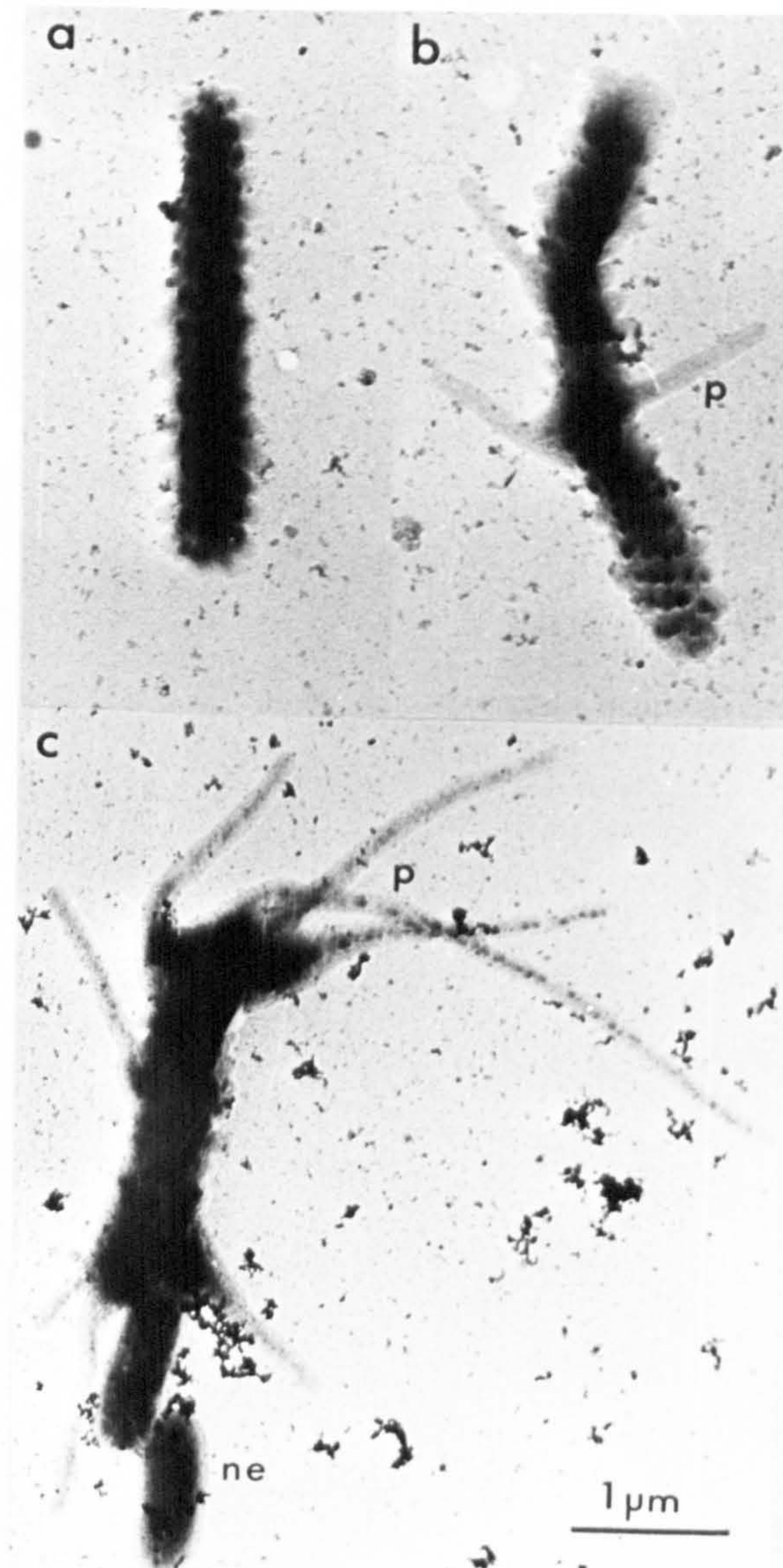


Fig. 2.41 Electron micrographs of D.1, 4 showing (a) nonexpressed form, (b) partially expressed form, and (c) expressed phenotype. Occasionally rod cells (ne) were also present. (p = prosthecae). (Gold/Palladium shadowed).

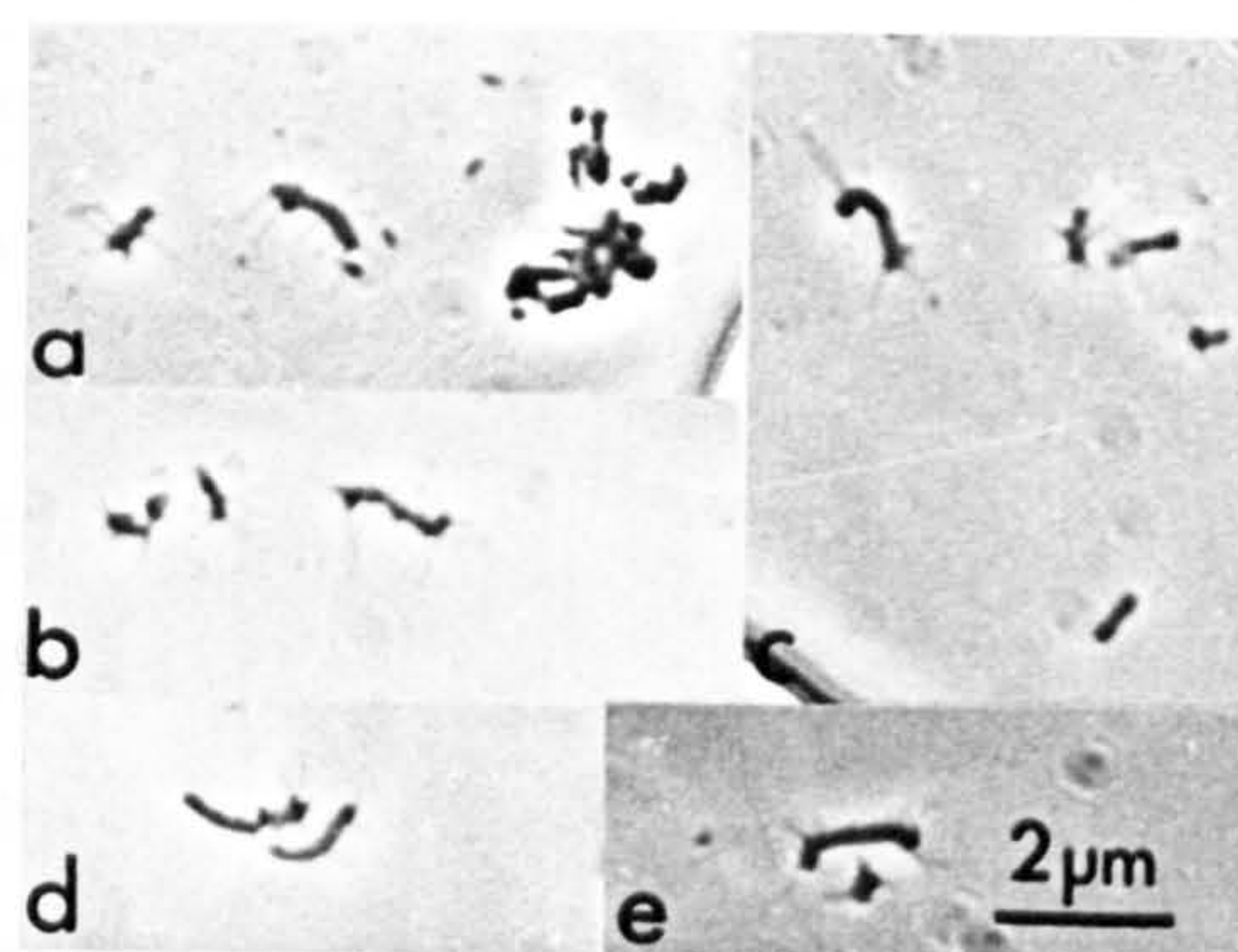


Fig. 2.42 Light micrographs of isolate D.1, 2, a presumptive Ancalomicrobium (Staley, 1968).

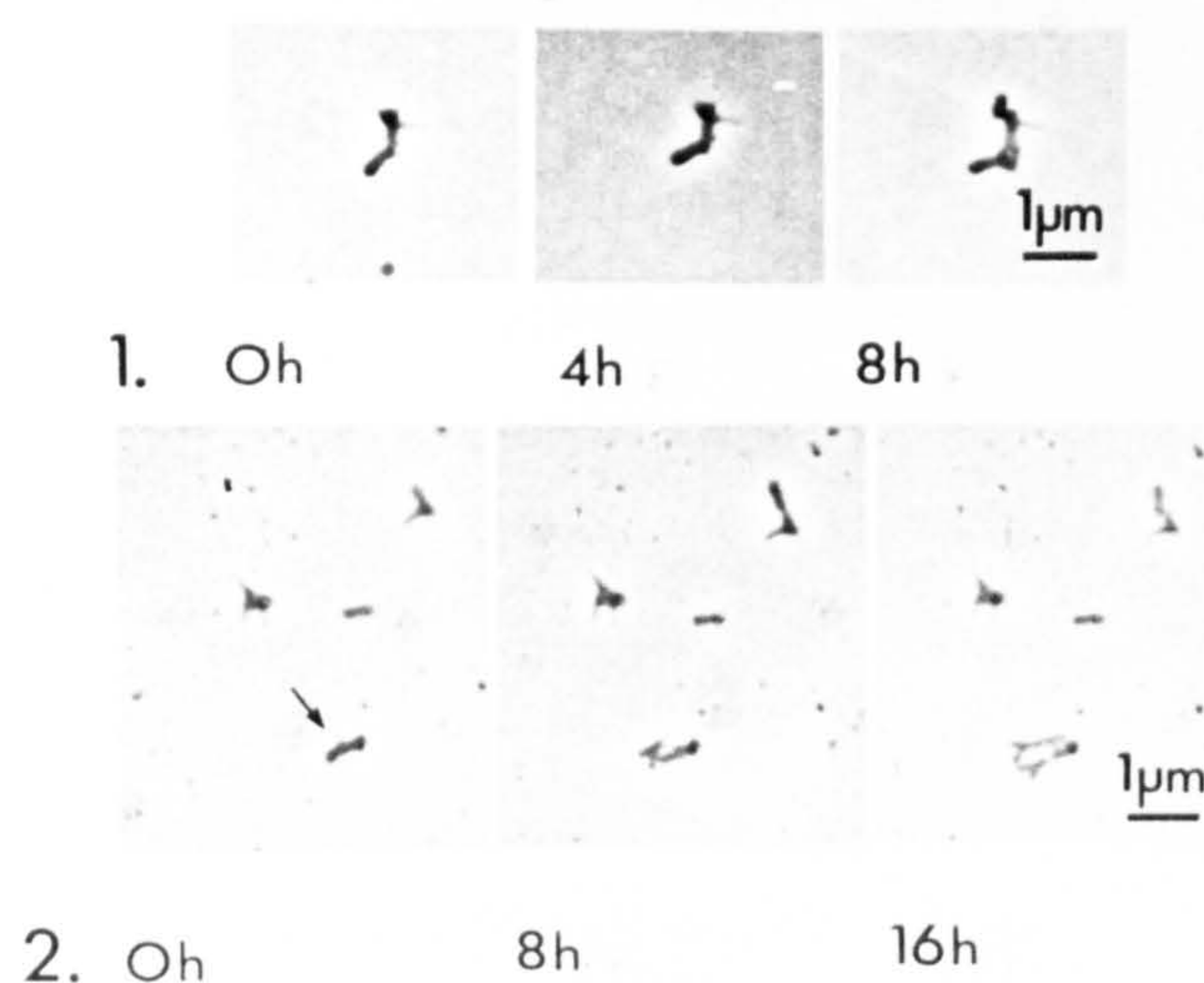


Fig. 2.43 Slide cultures of (1) D.1, 2 and (2) D.1, 4 showing budding mode of reproduction. Expressed forms gave rise to nonexpressed forms (arrowed), the phenotypic variations possibly being a consequence of the concentration of nutrients in the agar.

After two subcultures of D. 1, 2 into PWP medium, small rod-shaped bacteria appeared. Plating out from liquid media gave rise to two colony types, in approximately equal proportions. One colony type was beige and mucoid, resembling the parental colony morphology and was composed of multiappendaged cells, whilst the other colony type was paler with a granular surface, which grew deep into the agar and was shown to be made up from nonmotile rods, which possessed a unique and distinct morphology (Fig. 2.40, types 2 and 3). Recloning of either colony type gave the same result. Slide culture studies also showed that either cell gave rise to a mixture of both cell types, although the predominant cell type was always the parental cell, possibly a reflection on conditions of growth in slide cultures (Fig. 2.43). These results indicated that there was appendage induction or repression within an actively growing culture, with the possible 'triggering device' being the nutrient status of the cell's immediate environment. Further subculturing of D. 1, 2 eventually caused total repression of the multiappendaged cell form, despite exhaustive attempts to induce the expression of the appendages by variations in the culture medium (Section 2.II.3).

Isolate D. 1, 4

This isolate differed from previous isolates of multiappendaged bacteria in the number of prosthecae emanating from the cell body (Figs. 2.38, 2.44). Up to fifty prosthecae was not unusual for a mature cell, whose body was essentially rod-shaped with tapered ends. The surface of the cell body had a granular appearance, being composed of spherical structures, about $0.1\ \mu\text{m}$ in diameter, arranged regularly with several in a row, the rows forming a helical pattern (Fig. 2.41a, 2.46). The surface structure resembled observations made by Orenski *et al.* (1966a) on Helicoidal polyspheroides, an organism described as bearing spherical inflations, and by Nikitin *et al.* (1966) on Tuberoidbacter (Figs. 2.45, 2.46). The prosthecae of D. 1, 4 were never observed to bifurcate or branch, nor did they at any time show

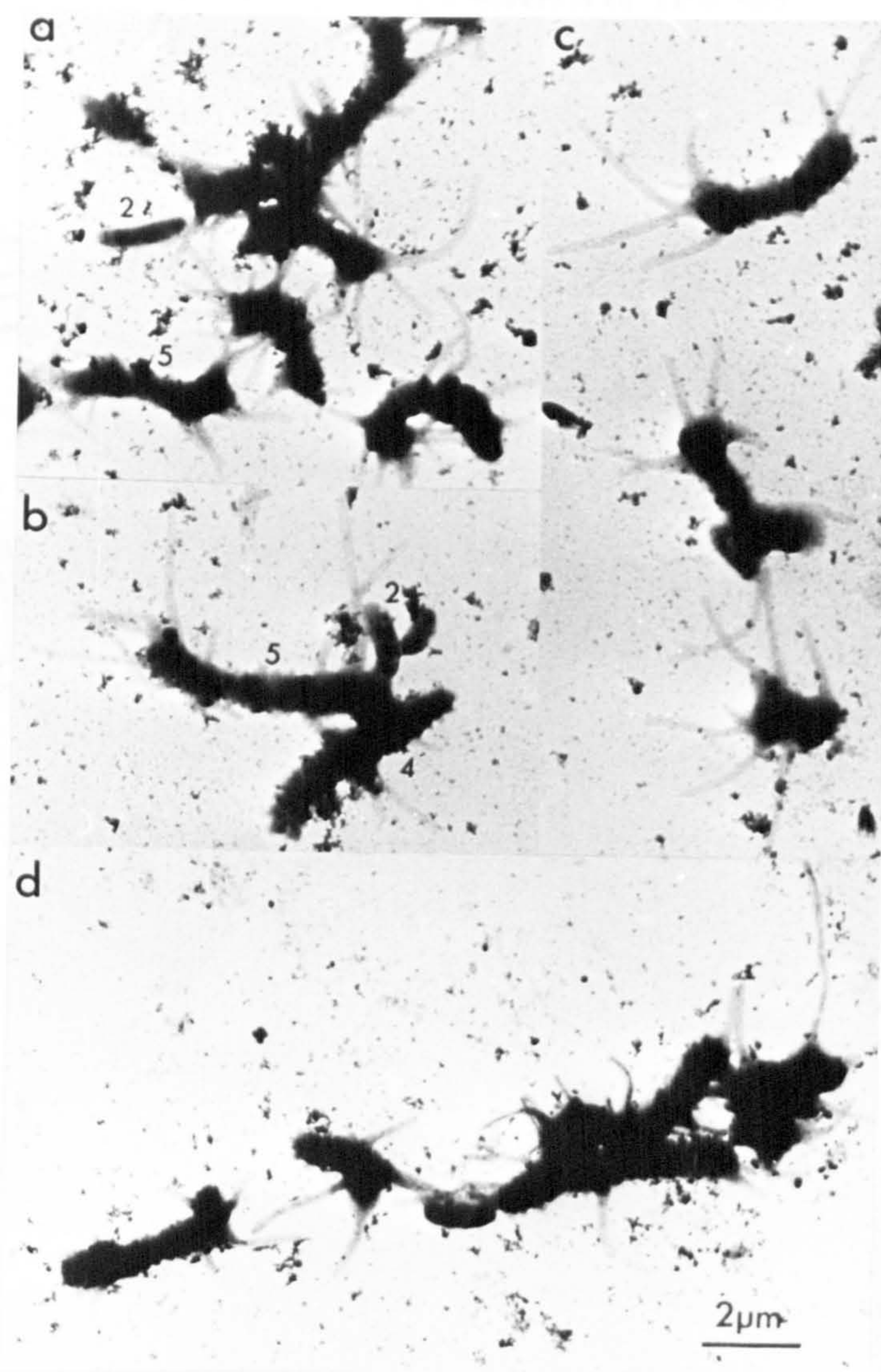
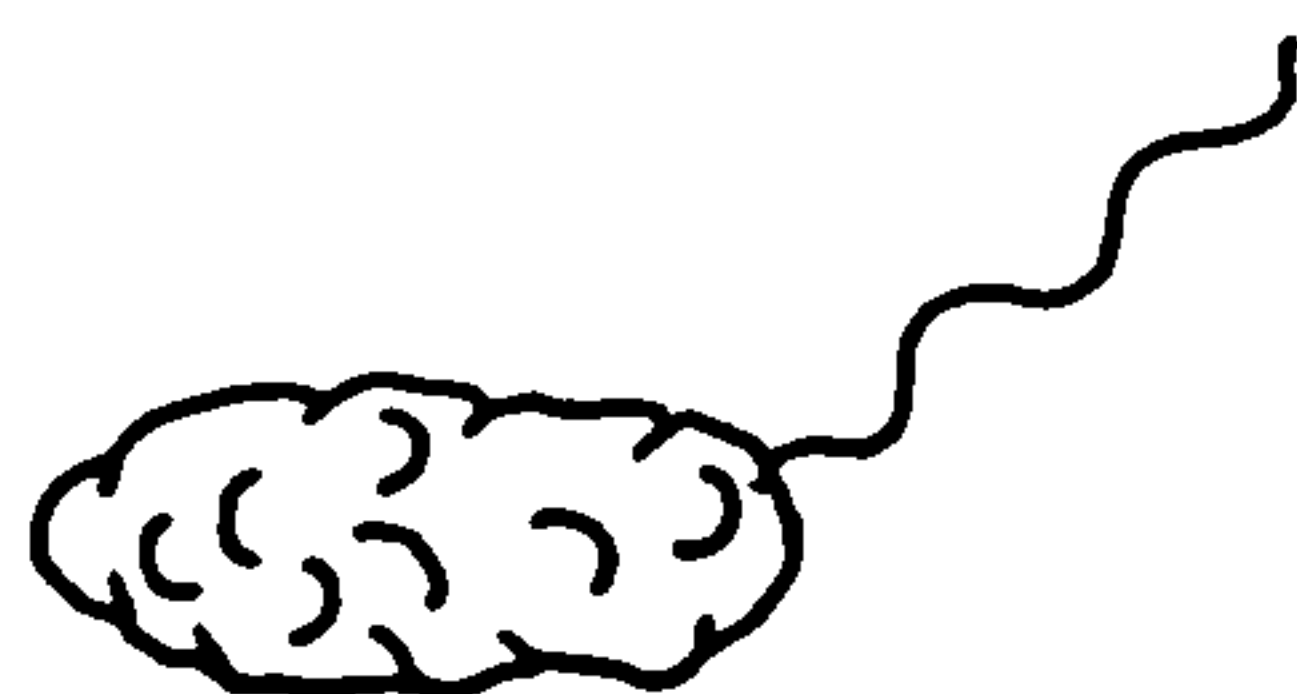


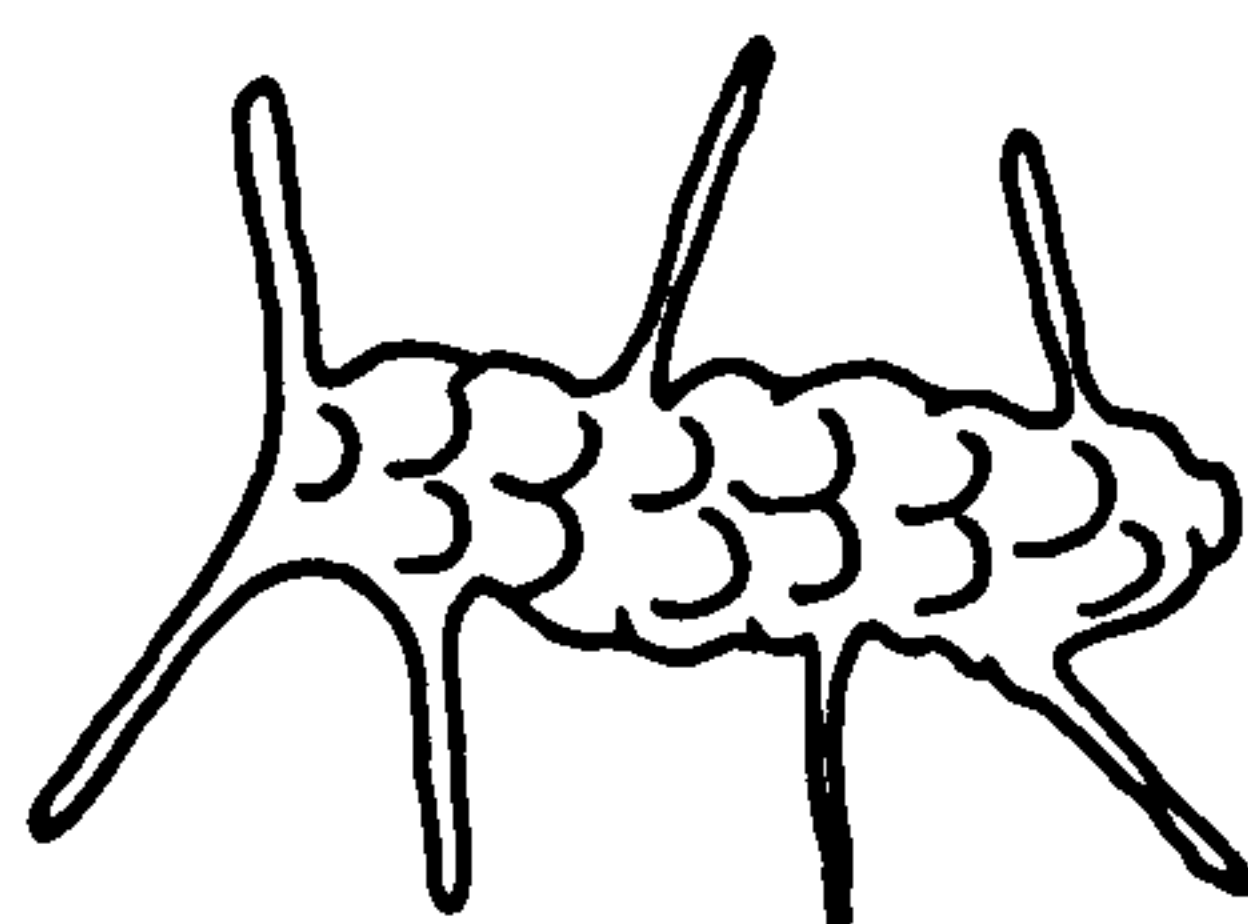
Fig. 2.44 Electron micrographs of isolate D.1,4 population, showing the mixed phenotype. Numbers refer to cell types outlined in Fig. 2.1.



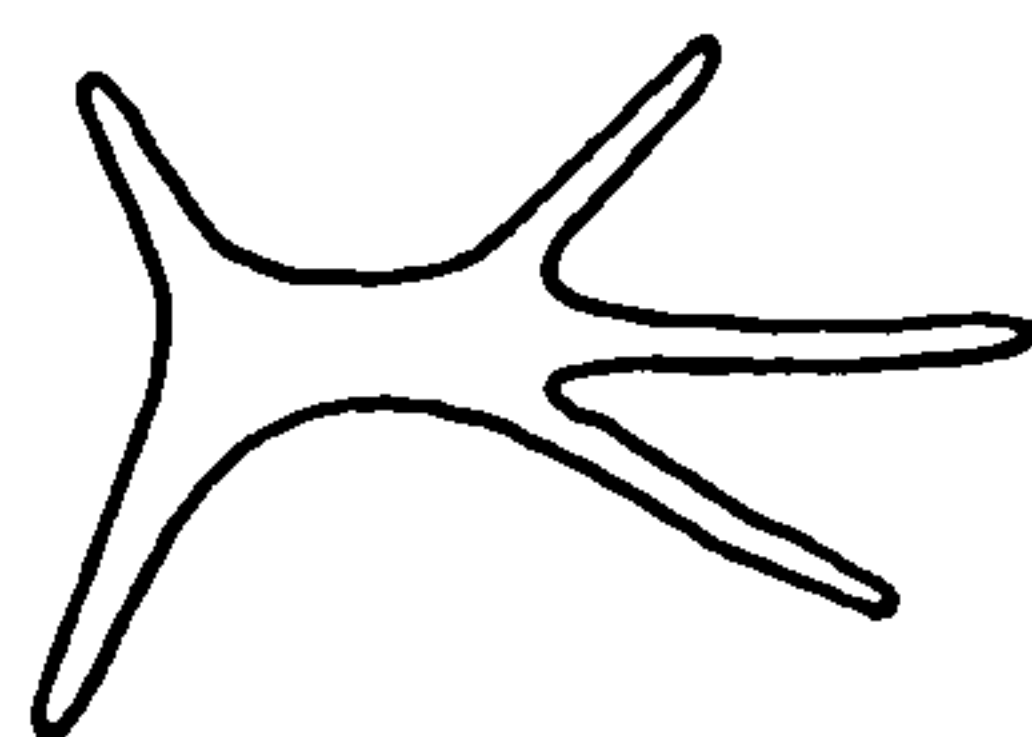
Agrobacterium
polysphaeroidum
(Nikitin, 1973)



Helicoidal
polysphaeroides
(Orenski et al, 1966a)



Tuberoideobacter
(Nikitin et al, 1966)



Ancalomicrobium
(Staley, 1968)

Fig. 2.45 Morphologically unusual bacteria, and the genera to which they have been attributed.

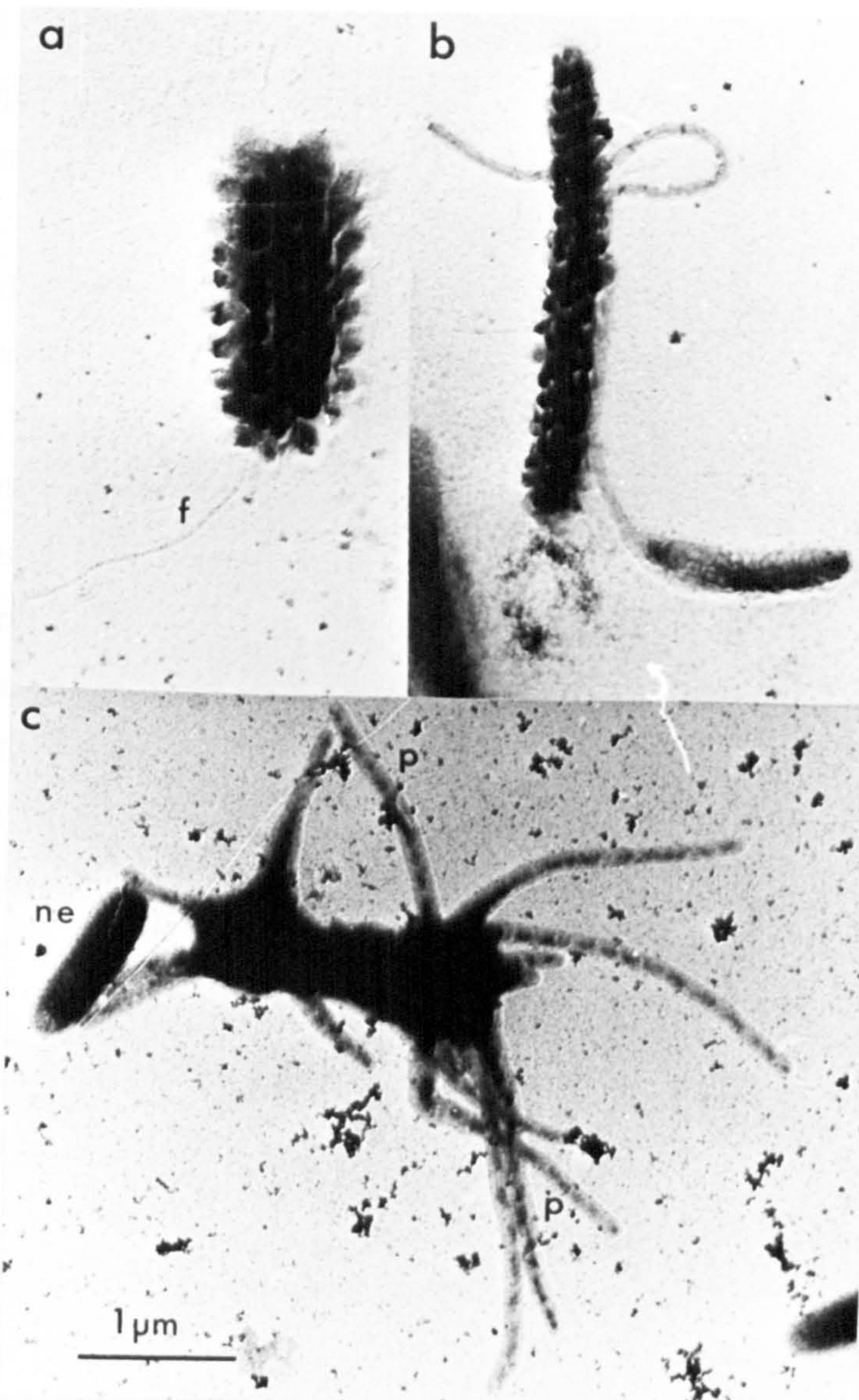


Fig. 2.46 Electron micrographs of phenotypes of a multiappendaged bacterium (D.1, 4), previously attributed to (a) Agrobacterium polyspheroidum, (b) Helicoidal polyspheroides, (c) Tuberoidobacter (see Text).

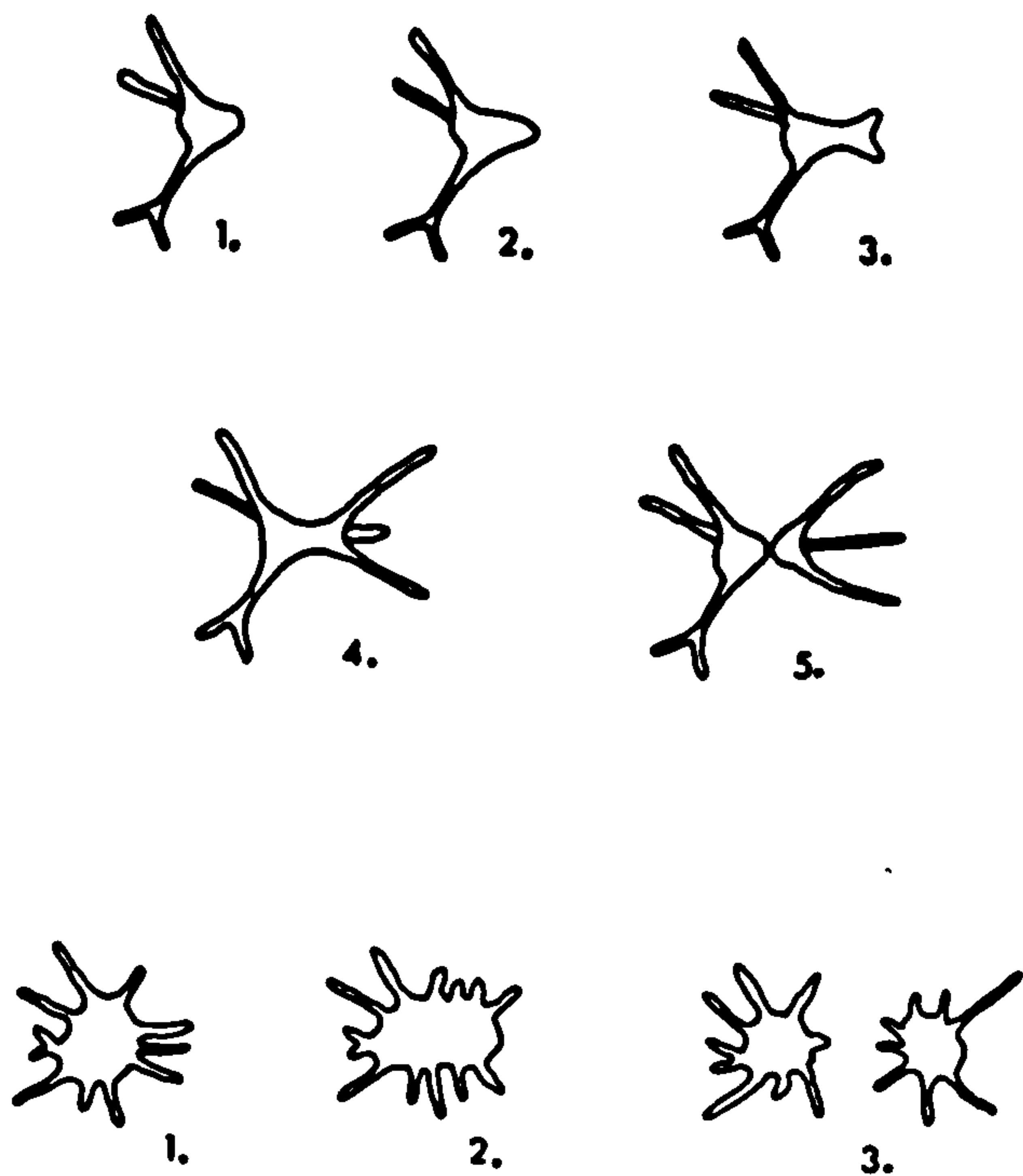
terminal swellings. Cells generally appeared to be nonmotile, and bore no holdfast structure. No vacuoles were observed.

Cells retained this morphology on PWP medium for several subcultures, however if they were inoculated into medium GY there was an extensive loss of appendages and the cells became very pleomorphic. Similarly, repeated subculturing of this organism caused the appendages to be only partially expressed, giving rise to complete mixtures of all types (Fig. 2.44) including rods with a granular appearance but lacking appendages (type 3), rods with some appendage expression (type 4) and rods with a full complement of appendages (type 5).

Dow et al. (1976) have shown appendage induction and repression in an isolated strain of Ancalomicrobium, in response to organic nutrient concentrations and to varying concentrations of phosphate, which caused drastic alterations to appendage length and number and to cell morphology, as the cells became very pleomorphic and concomitantly non-viable. Shapiro (1976) has shown that low phosphate levels caused drastic increases in the length of the Caulobacter stalk, however the stalk possesses crossbands which are said to be solid structures which would present an obstruction to any uptake function, although the non-permeability of the crossbands has not been shown. It has been proposed that the Caulobacter stalk, with the membranous organelle at the base, has evolved to a high degree of efficiency, to become a highly organised uptake system (Dow, 1974), where possibly the crossbands play an integral role in the function as well as the structure. The appendage of Ancalomicrobium sp. and other multiappendaged bacteria may well lack such sophistication, and consequently to obtain similar uptake capabilities, these organisms possess several inducible appendages, as illustrated by D.1, 2 and D.1, 4.

Life cycle

Both isolates reproduced by budding, as determined by slide culture studies (Figs. 2.43, 2.47), the appendages are not involved in the reproductive process. The buds were formed from one end of the cell, and as they developed, two or more prosthecae differentiated from the bud. Division occurred transversely when the mother and daughter



Flg. 2.47 Budding mode of reproduction in multiappendaged Anca lomicrobium (upper) and binary fission in Prosthecomicrobium (lower) (after Staley, 1968).

cells had attained approximately the same size (Fig. 2.48).

Ultrastructure

Thin sections confirmed that the appendages of D. 1, 2 and D. 1, 4 are cellular extensions as defined by Staley (1968). The prosthecae appeared to contain cytoplasmic material, however overall detailed structure was poorly resolved (Fig. 2.49). The unique and distinctive subunit array of the cell wall of the multiappendaged species under normal culture conditions suggests that appendage production is synonymous in origin with protuberances (Fig. 2.50). Under high organic nutrient levels, the cell wall structure was solely characterised by undulations which were continuous with the cell wall surface layers (Fig. 2.49). No internal structures were observed (cf. Caulobacter, Cohen-Bazire et al., 1966) and no internal compartmentalisation was evident.

Phenotypic variation

In addition to the multiappendaged cell type and the nonexpressed cell forms seen in cultures of both D. 1, 2 and D. 1, 4, other cell types were observed. Frequently smaller rods were found in cultures rich in organic nutrient, which closely resembled the nonexpressed rod form, in their granular surface appearance, although these smaller rods possessed fewer protuberances and some were motile by a single subpolar flagellum. This cell type closely resembled Agrobacterium polysphaeroidum, as described by Nikitin (1973) (Fig. 2.46a).

Small motile cells could also be observed in liquid cultures, high in organic nutrients ($>200 \mu\text{g/ml}$); these cells have been previously reported (Dow, 1974). The cells were ovoid and non-appendaged (Fig. 2.46c). After prolonged incubation, the cells developed a granular cell wall appearance, and ultimately protuberances formed. It is assumed that these cells bud off the preappendaged cells. Fig. 2.48 gives a summary of the proposed life cycle of multiappendaged cells described here. Both these isolates are characterised by several morphological forms; this may well be a common property of this unusual group of bacteria.

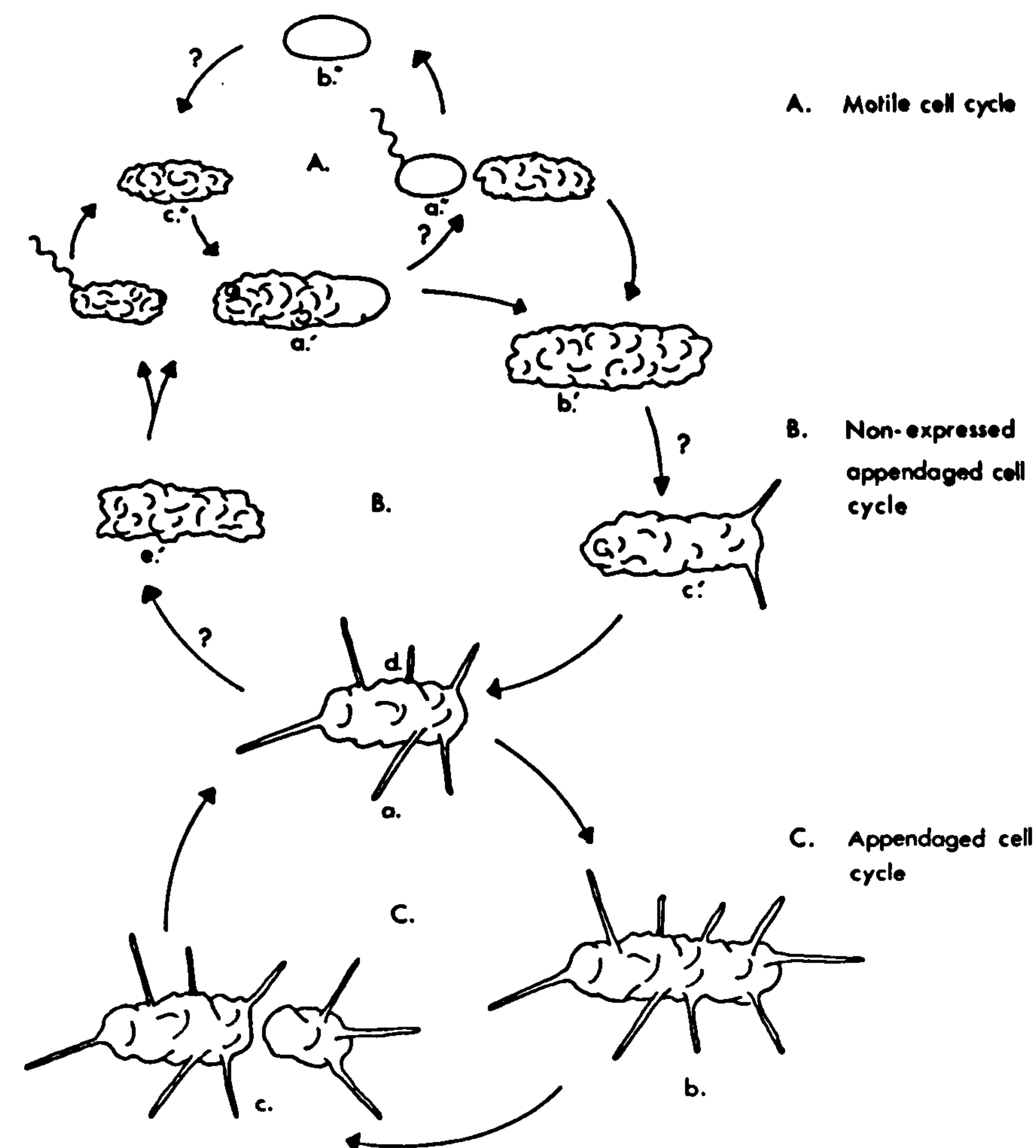


Fig. 2.48 Proposed life cycle of *Acanthamoeba*, illustrating the motile life cycle (A), the nonexpressed cell cycle (B) and the classic appendaged cell cycle (C). It is proposed that environmental conditions effect phenotypic variations. Appendaged cell cycle is induced in medium low in organic nutrients ($< 100 \mu\text{g/ml}$), whereas the nonappendaged cell cycle occurs in medium containing in excess of $175 \mu\text{g/ml}$. Stimulating factors for the motile cell cycle were not determined.

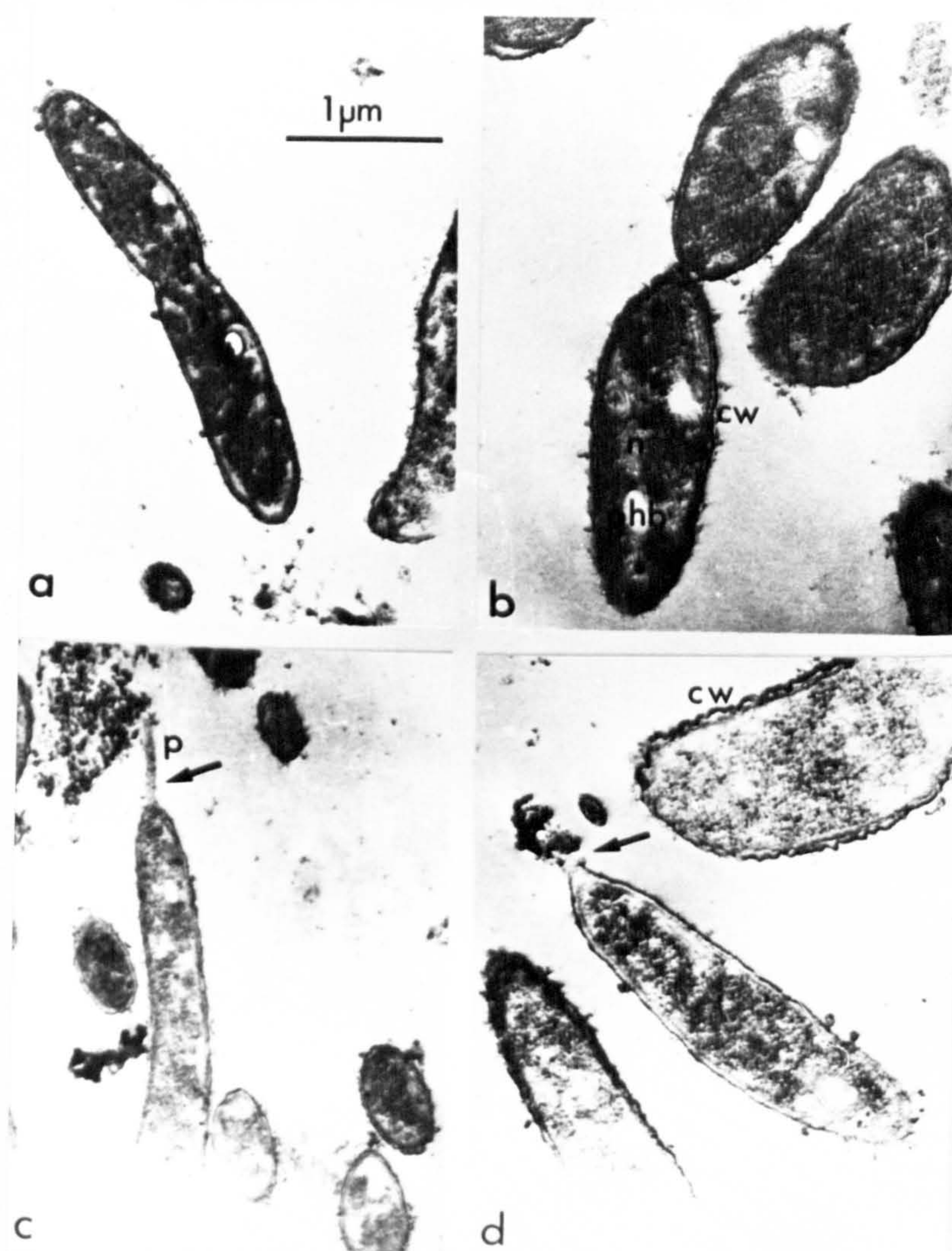


Fig. 2.49 Ultrathin sections of multiappendaged bacterium D.1, 4. (a), (b) nonexpressed forms, (c), (d) appendage formation, (p) arrowed. Note undulations in cell wall (cw) in (d). (phb = poly β -hydroxybutyrate, n = DNA, cw = cell wall, p = prostheca).

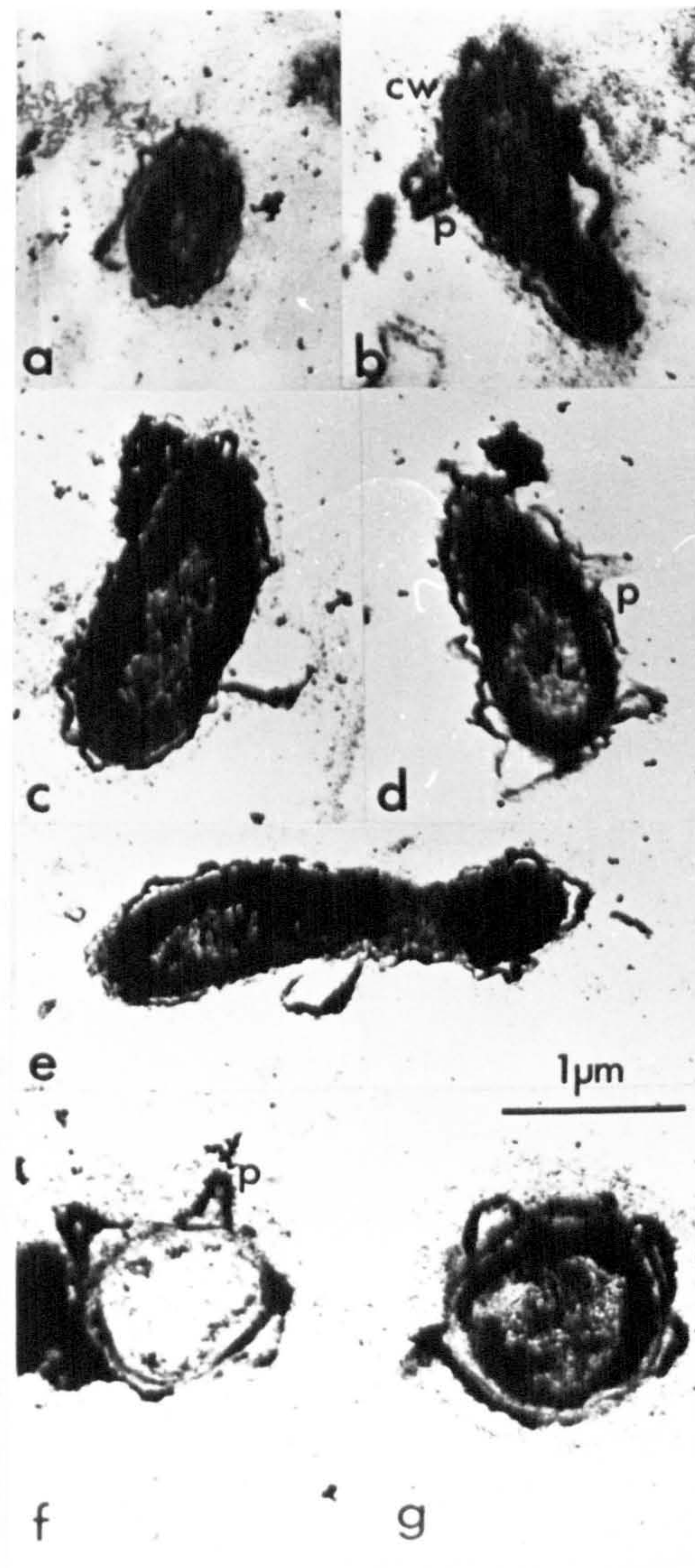


Fig. 2.50 Ultrathin sections of multiappendaged bacterium, D.1,2 , illustrating the continuity of appendages with cell wall in the transverse sections [(a), (f) and (g)].
(p = prostheca, cw = cell wall, n = DNA)

Of the two isolates described, D. 1, 2 closely resembles Ancalomicrobium (Staley, 1968). D. 1, 4, however, cannot be accommodated into any existing genus description, due to its number of inducible appendages and its phenotypic nature. The ability of the appendages of D. 1, 4 to be induced or repressed by environmental stimuli appears to be analogous to the observed loss of appendages by an Ancalomicrobium sp. studied by Dow et al. (1976). D. 1, 4 could be accommodated in the genus Ancalomicrobium if it was redefined to accommodate bacteria with large numbers of prosthecae, capable of morphological variation in response to environmental stimuli. This would then remove the problem of different phenotypes being assigned to different genera (Fig. 2.45).

Re-examination of water samples in enrichments in the light of this study on phenotypic variation shows that there are still many different types of multiappendaged bacteria to be isolated and studied, but their diversity may not be as pronounced as once thought (Bystricky, 1970).

(c) Pedomicrobium Introduction

In determining which microbial species were responsible for the deposit of heavy metals from the environment, Aristovskya (1961) isolated a microorganism which consisted of round, oval and, less frequently, elongated cells, 0.4 - 2.0 μm long, joined together by branched threads. Multiplication was by budding from the tips of the threads, although sessile budding was observed. The isolated organism resembled Rhodomicrobium vannielii in morphology and reproduction as described by Duchow and Douglas (1949) and was named Pedomicrobium. This bacterium appeared widespread in soil, and could deposit manganese or iron, apparently by oxidising them to the corresponding oxides. Aristovskya also found Metallogenium, as described by Perfil'ev and Gabe (1964) in soil deposits, when attempting to culture Pedomicrobium sp. on organomineral complex media.

During regular screening of freshwater enrichments from oligotrophic water bodies, an organism closely resembling Pedomicrobium has been observed and isolated in pure culture (Fig. 2.51).

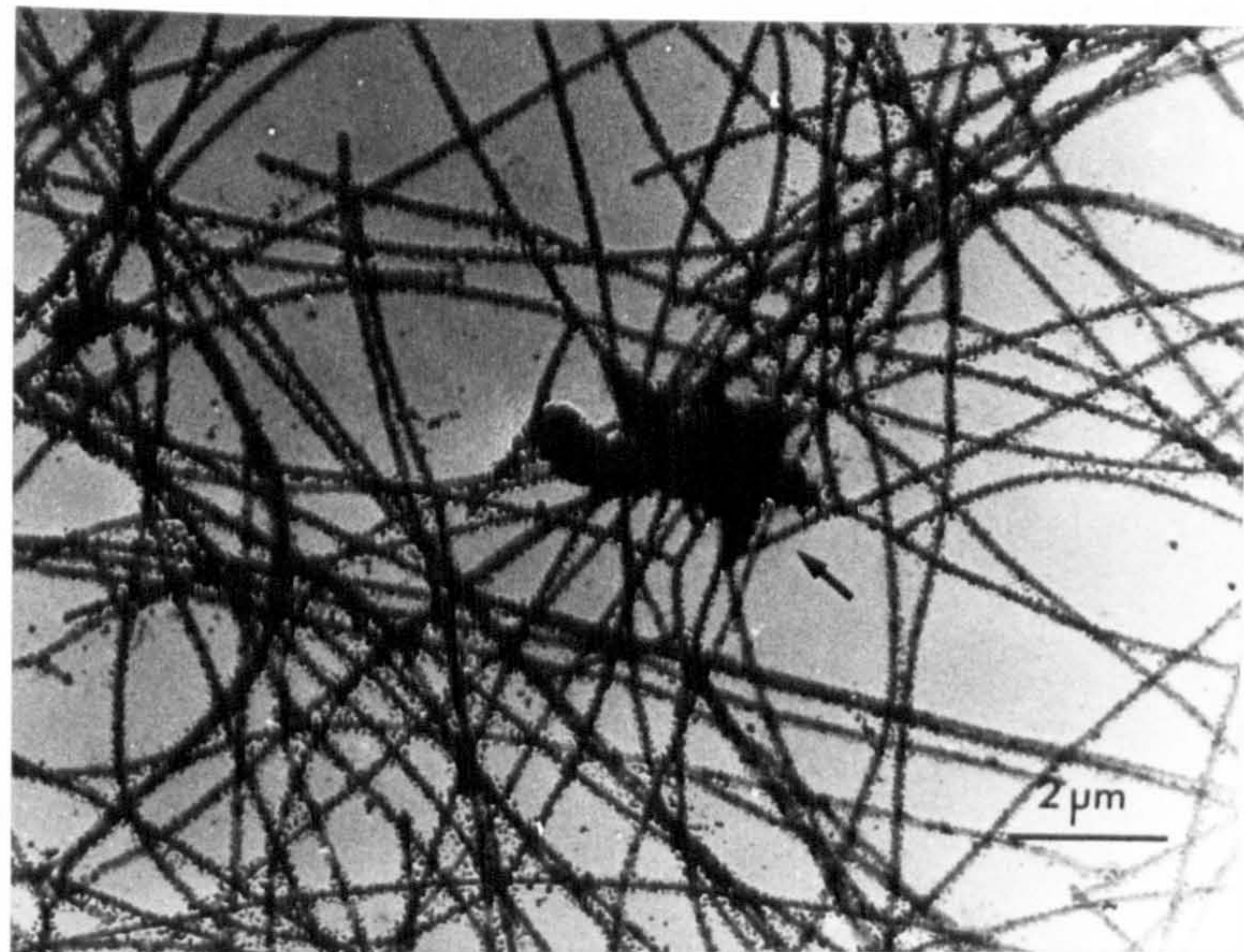
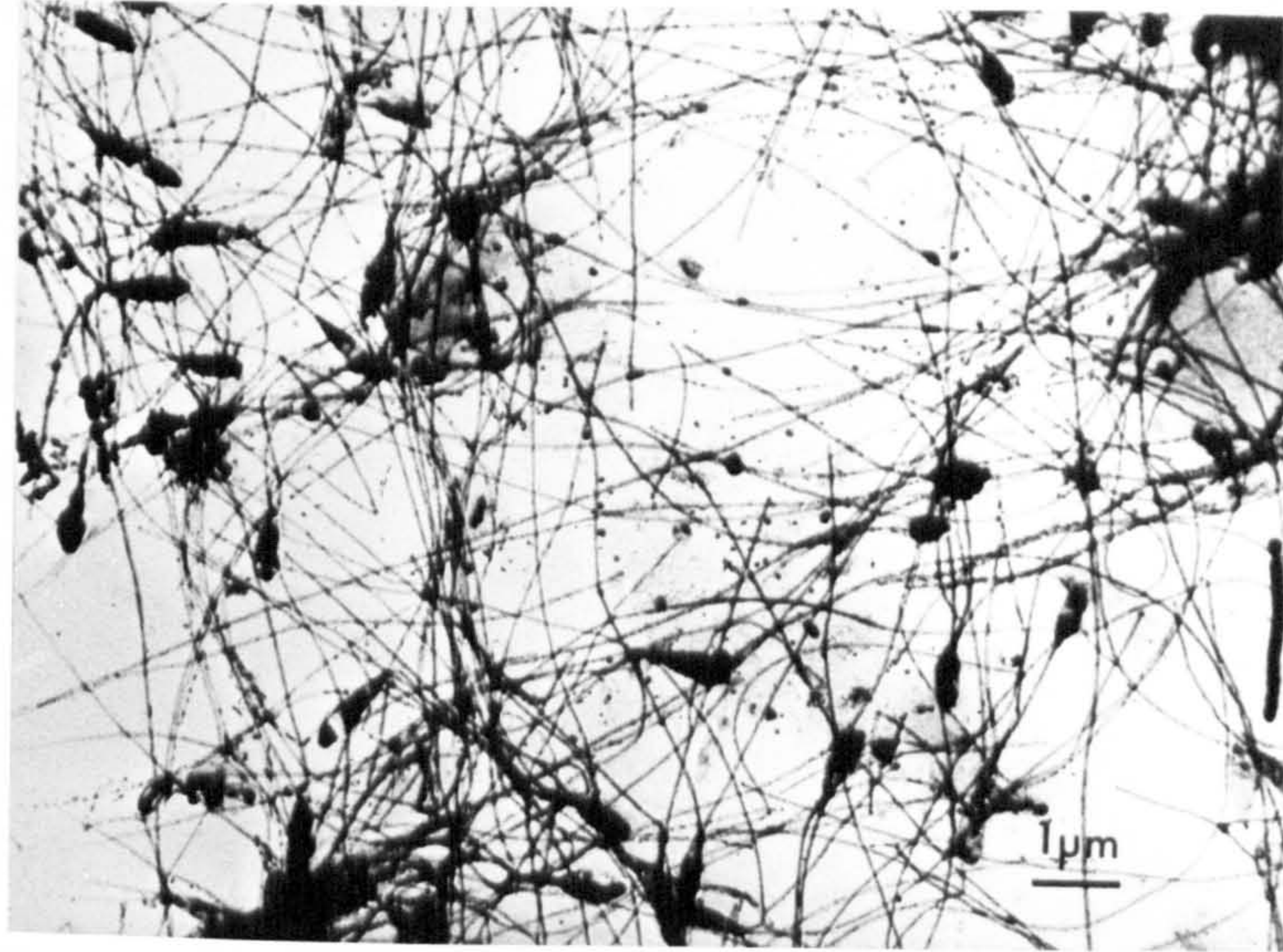


Fig. 2.51 Electron micrographs of cellular matrices in the natural environment, composed of cell bodies (arrowed) and extensive prosthecal networks.

Results and Discussion

Freshwater isolate - morphology and physiology

After 8 weeks incubation, on medium HB and C, small brown colonies developed, growing deep into the agar. Colonies were composed of ovoid shaped cells with hyphae of diameter 0.15 - 0.3 μm , and of variable length, extending from several sites on the cell surface (the hyphae being extensively branched to form complex networks, (Fig. 2.52). Small swellings or buds could be observed at the hyphal tips; intercalary buds have also been observed (Figs. 2.53, 2.54). The addition of 0.05% (w/v) manganese chloride to the medium caused the colonies to darken, and deposits were observed on the cell bodies and filaments (Fig. 2.54). This isolate grew well on HB medium which was used for routine culturing under aerobic conditions, at 30⁰ C. After several subcultures, the cell complexes were considerably reduced but with most of the cells remaining multi-appendaged (Fig. 2.56).

Life cycle

Multiplication was primarily by budding at the tips of the cellular prosthecae (-hyphae, Aristovskya, 1958). Daughter cells either remained attached to the hyphae or separated to form swarmer cells, motile by a single polar flagellum. After undergoing a maturation stage, when the cell increased in size and lost its flagellum, mature swarmer cells grew from one to numerous hyphae from several sites on their cell surface (Fig. 2.56a). The hyphae then developed into extensive networks with further budding (Figs. 2.56b, c, 2.58). Intercalary buds were observed (Fig. 2.57b) as was division of mother cells (Fig. 2.60).

Pleomorphism

This aquatic type of Pedomicrobium morphologically resembled hyphomicrobia when grown on mineral media with methylamine hydrochloride and mineral media with manganese and/or iron salts supplements (Section 3.III.4). In the natural environment, and

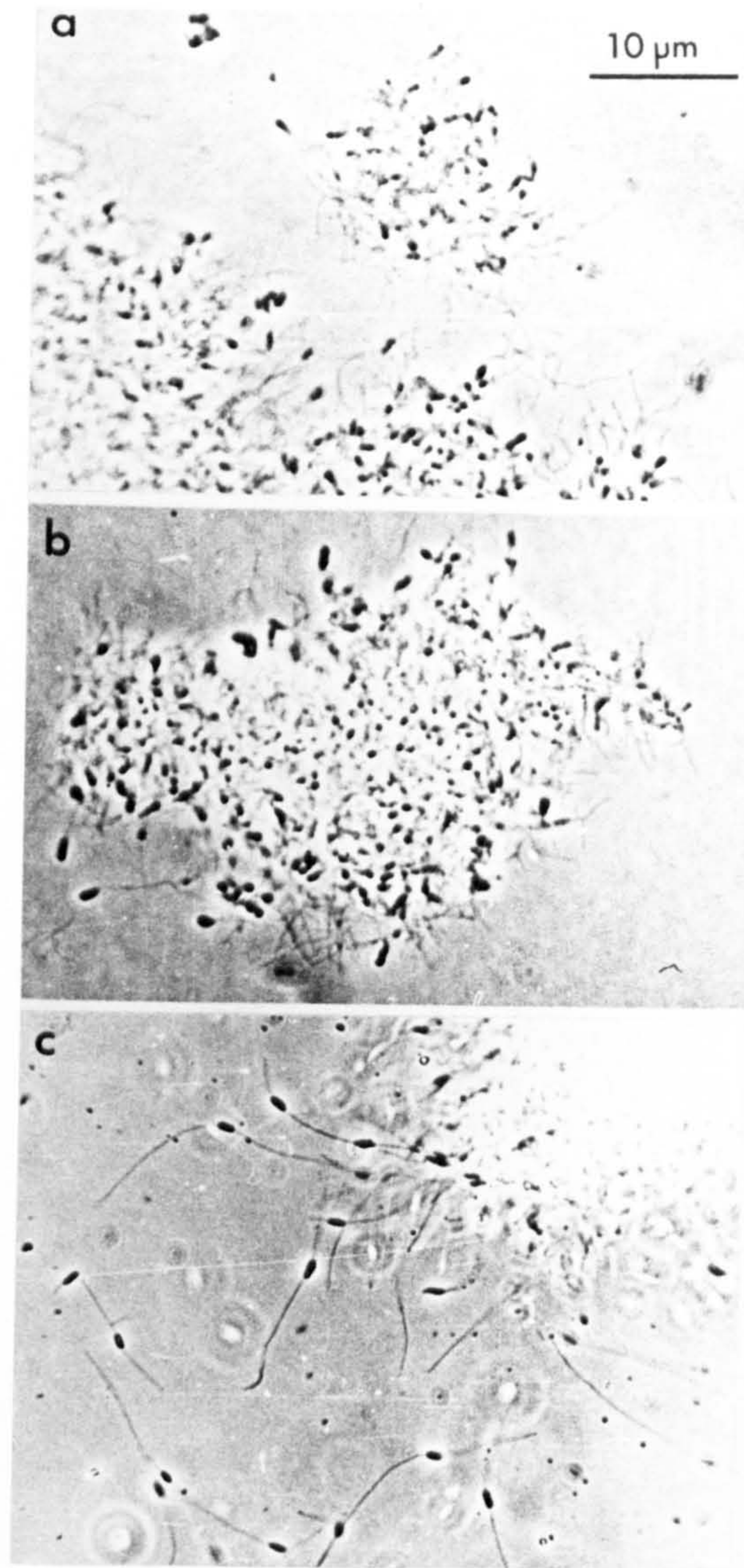


Fig. 2.52 Light micrographs of complex networks of cells, from the pellicle formed in 'static' enrichments. Colonies were composed of ovoid cells, with prosthecae.

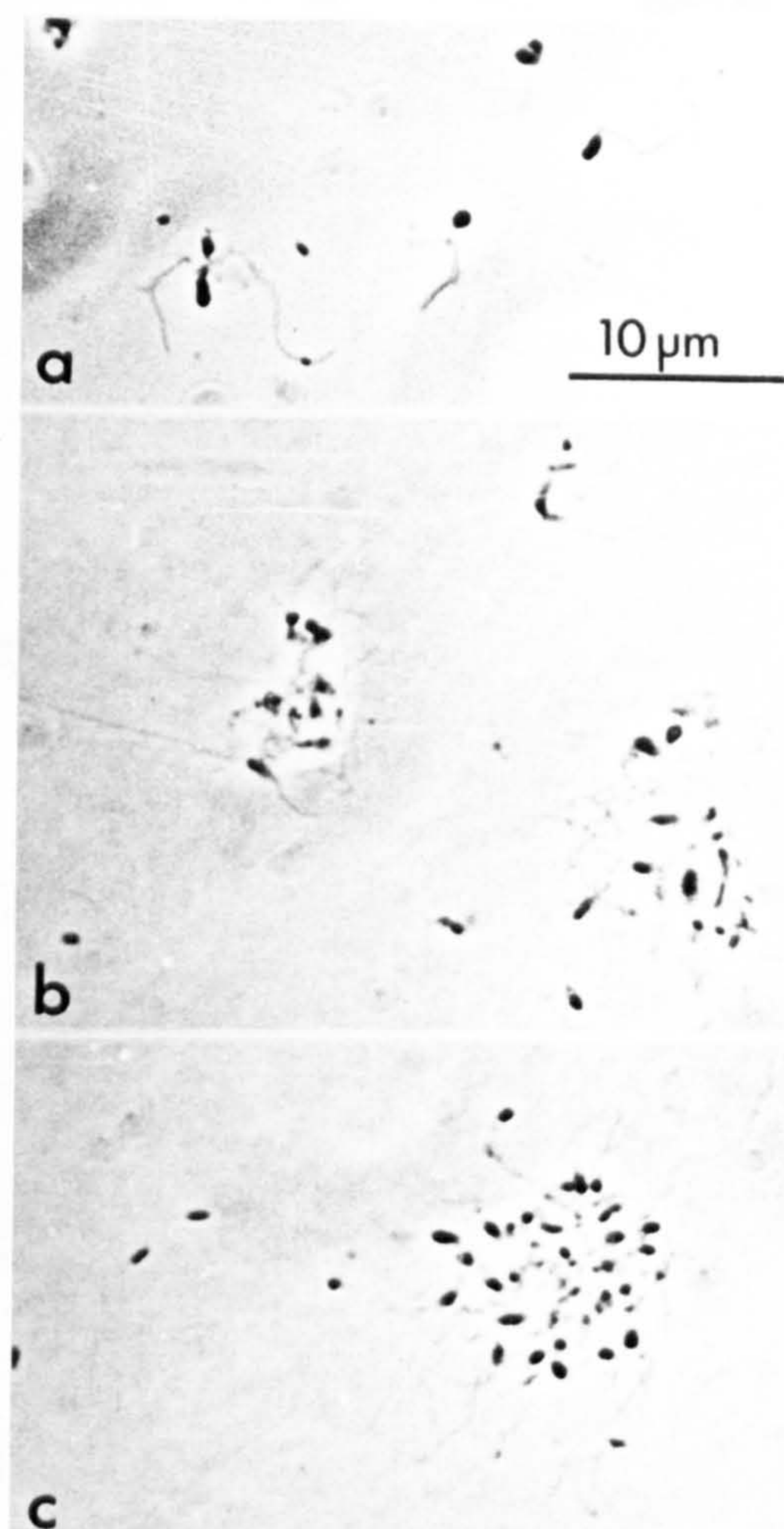


Fig. 2.53

Cell aggregates from colonies obtained from the pellicle. Cell shape was poorly defined under the light microscope, due to deposits on the cell bodies and stalks.

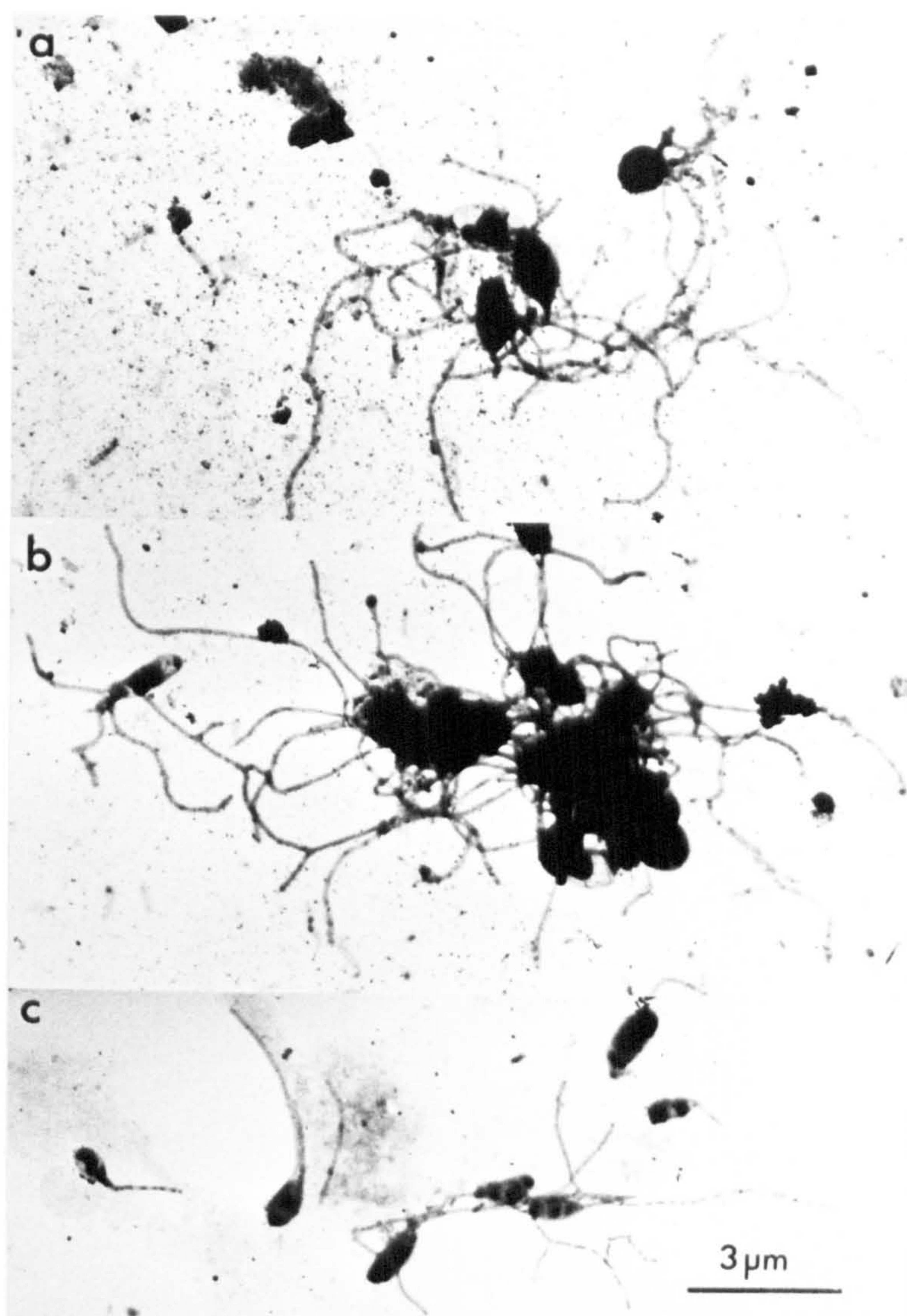


Fig. 2.54 Electron micrographs of cells, growing in medium supplemented with manganese salts, were multi-appendaged. The appendages were frequently branched. Deposits, presumably of manganese oxides, masked the cell bodies.

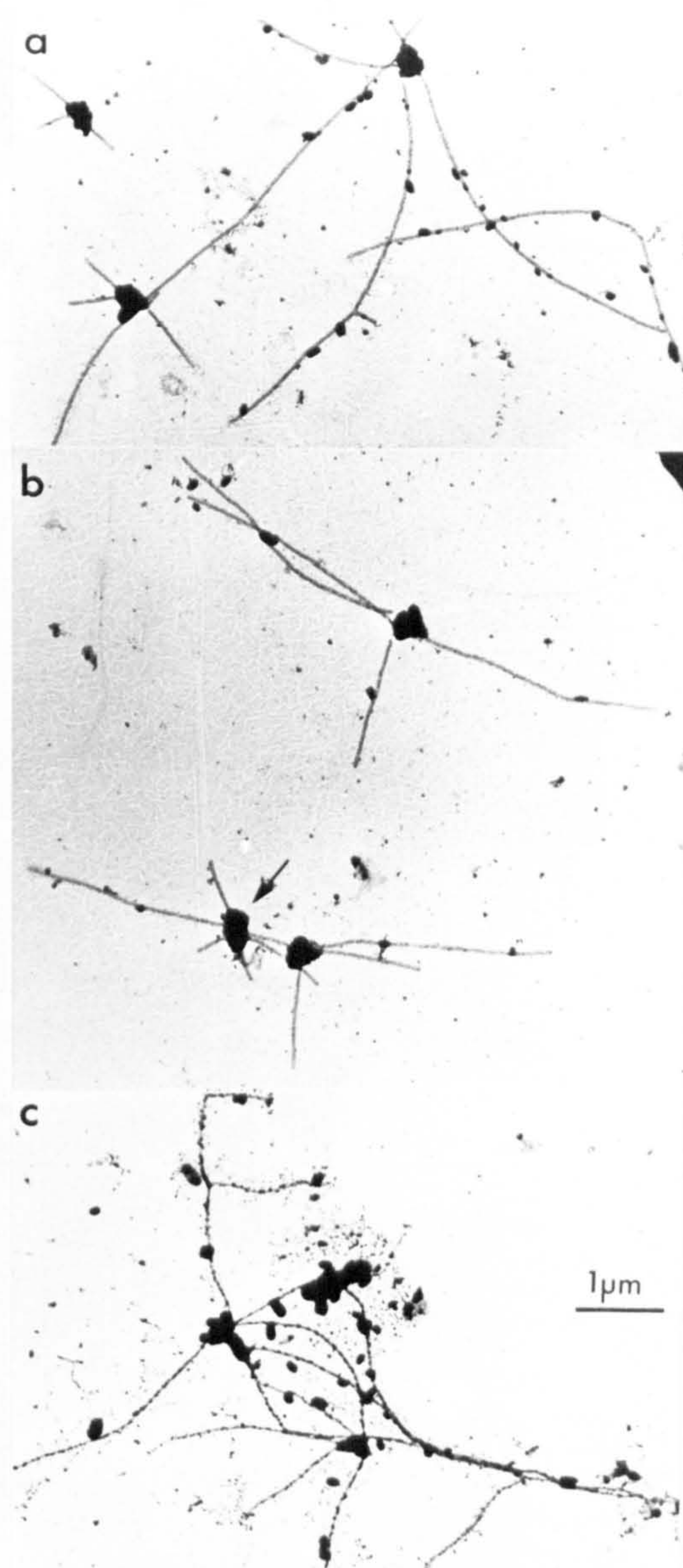


Fig. 2.56 Pure isolate of presumptive Pedomicrobium growing in culture. Prosthecae extended from several locations on the cell body (a), (b) (arrowed), and developed into multi-cellular arrays (c). (Gold/Palladium shadowed).



Fig. 2.57

1. Electron micrographs of cell bodies and stalks within the multi-cellular array of Pedomicrobium. Stalks branched extensively, cell bodies (arrowed) were infrequent. (Gold/Palladium shadowed).

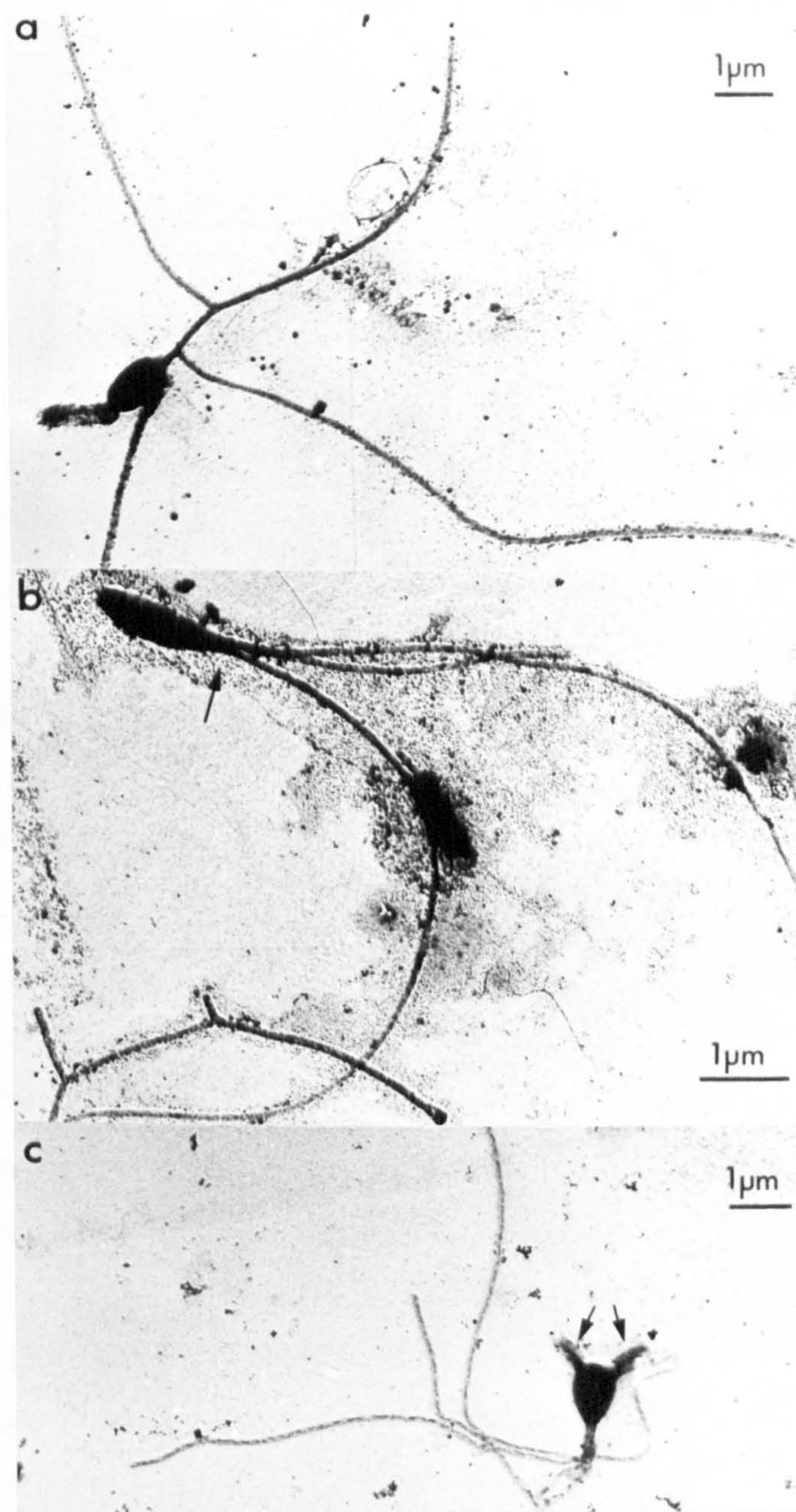


Fig. 2.58

2. Electron micrographs of cell bodies and stalks within the multi-cellular arrays of Pedomicrobium. Several stalks occasionally emerged from one pole (b) or from several locations on the cell body, arrowed (c). (Gold/Palladium shadowed).

especially in the freshwater "natural" enrichments set up in the laboratory, this isolate resembled the classic descriptions of Pedomicrobium (Aristovskya, 1961, 1963) (Fig. 2.59). Although species of Pedomicrobium closely resembled Hyphomicrobium they differed in their ability to deposit oxides of manganese and iron (Zavarzin, 1968). Studies by Bauld, Tyler and Marshall (1971) on an isolate of Hyphomicrobium, designated T37, have shown that this organism can have a classic life cycle of swimmers and stalked mother cells, but is also capable of a more complex multicellular or colonial morphology (Fig. 2.59) (see Section 3.III.4). Similar observations have been made by Hirsch and Conti (1964), Tyler and Marshall (1967b) and Hirsch (1968). The factors dictating swarm cell retention and hence colony formation are not known, although the nutrient status of the environment has been implicated (Tyler and Marshall, 1967b). These workers regarded Pedomicrobium as but one expression of a pleomorphic Hyphomicrobium, a contention supported by Bauld et al. (1971). Hirsch (1974) disagrees, claiming that in Pedomicrobium hyphae grow out from one to several points, whereas in Hyphomicrobium hyphae grow from the poles only. However, this study supports the findings of Tyler and Marshall (1967b) in that this isolate behaves as typical Hyphomicrobium or Pedomicrobium, depending on conditions of growth (see Section 3.III.4). The variable morphology of this organism, which is considered to be Hyphomicrobium, questions the credibility of the genus Pedomicrobium as described by Aristovskya (1961). (See Section 3 for supportive evidence for this contention).

(d) Metallogenium Introduction

Metallogenium, named for its ability to produce metal deposits in the natural environment, was observed by Perfil'ev and Gabe (1961) after an exhaustive search since 1937 for microorganisms involved in metallogenic processes. Due to the layers of manganese oxide masking the cellular structure, they named their isolate M. personatum. In

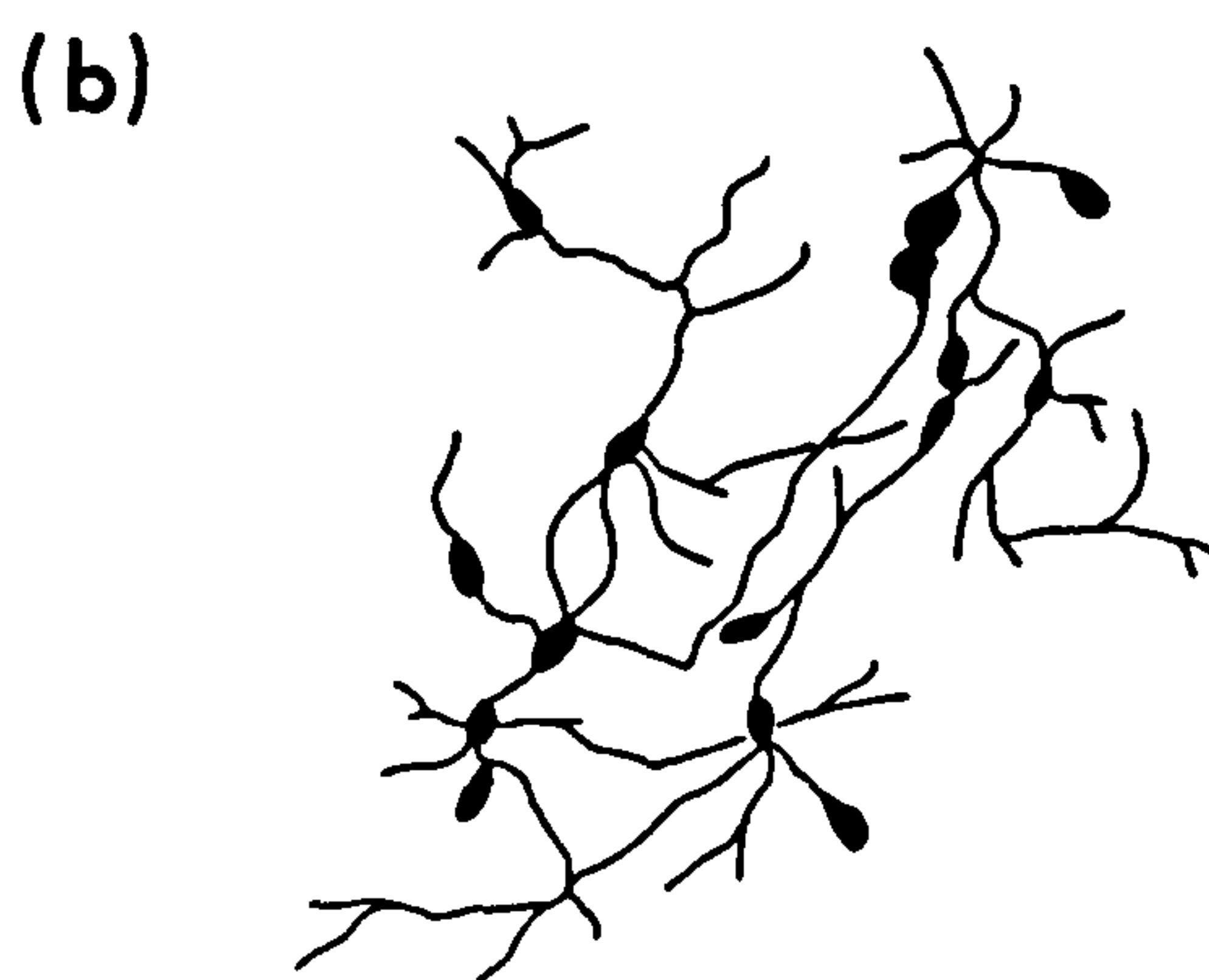


Fig. 2.59 Classic descriptions of (a) T 37 (Tyler and Marshall, 1967) and (b) Pedomicrobium (Aristovskya, 1961).

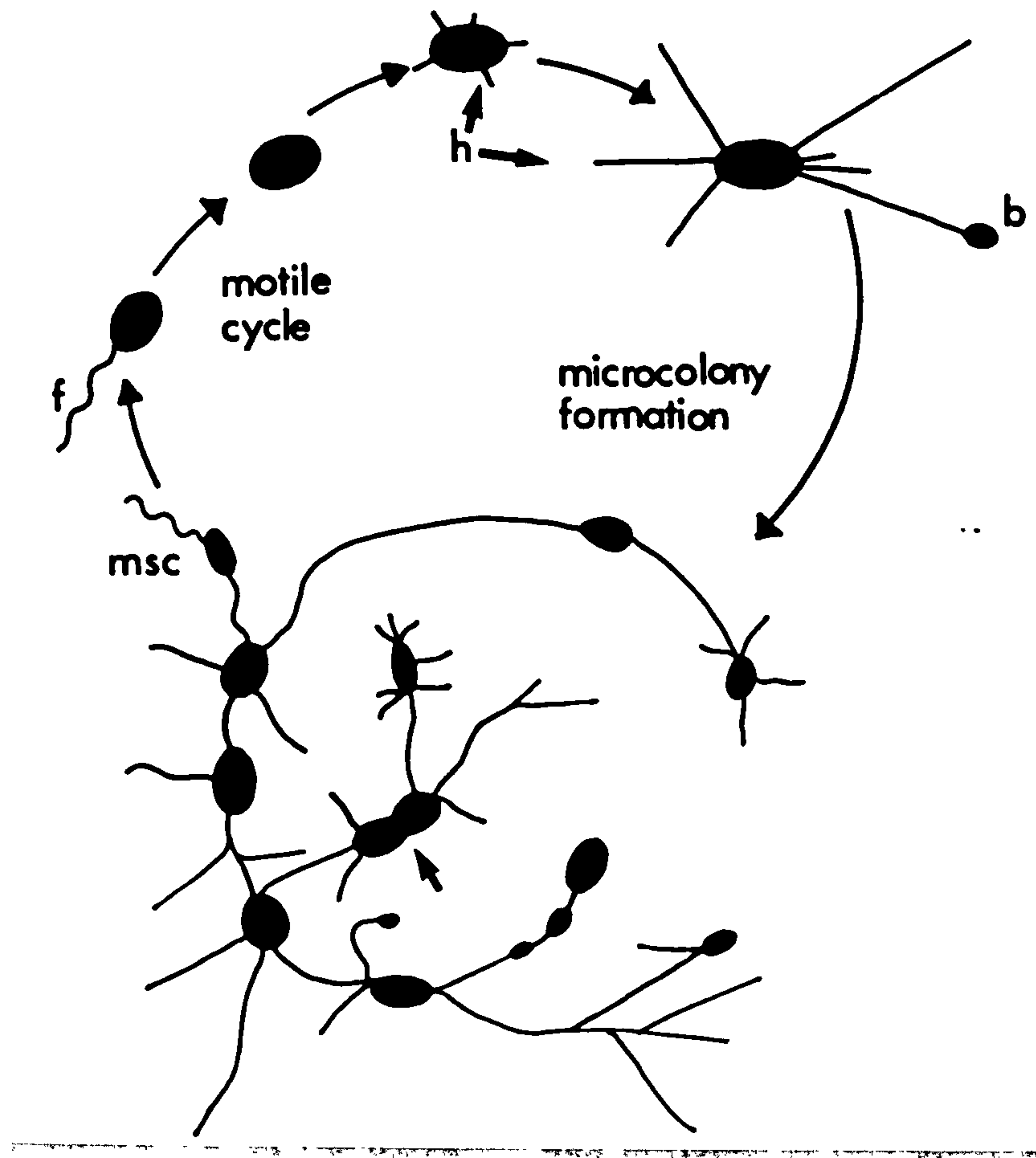


Fig. 2.60 Presumptive life cycle of Pedomicrobium, from studies of isolates obtained during the course of this study. The swarmer cell matures to develop prosthecae (h) from several locations, and finally develops into a multicellular array, from which new swimmers may be released (msc). Sessile budding may also occur (arrowed).

the same year, Zavarzin isolated a metal depositing microorganism which appeared to grow in the presence of bacteria or fungus, and named it M. symbioticum (Fig. 2.61). More recently, Dubinina (1973) has studied in detail the life cycle and pleomorphic nature of this organism and has proposed how its unusual development cycle contributes to its ability to retain and bind manganese oxides on various surfaces (Fig. 2.62).

Life cycle

Perfil'ev and Gabe (1961) described four different stages of the life cycle: a unicellular motile stage, a multicellular stage with radial filaments, and two cell forms encrusted in metal oxide deposits. They stated that multiplication occurred by the round reproduction cells which formed at the tip of the filaments. Zavarzin (1961) has also shown that reproduction is probably by the budding off of round cells from the filament tips. These cells are initially motile but they soon adhere to a substrate whereupon filamentous outgrowths appear on the cells (Fig. 2.62).

Manganese oxidation

Previous workers (Perfil'ev and Gabe, 1961; Zavarzin, 1961, 1963, 1964) have shown that Metallogenium oxidises divalent manganese to manganese dioxide. No evidence was found for this serving as any energy source. After the oxidation was initiated by this organism, it probably proceeded chemically. The mechanism for bacterial oxidation of manganese has not been determined.

Results and Discussion

Frequent observations were made during the course of this study of microorganisms resembling published descriptions of Metallogenium (Perfil'ev and Gabe, 1961; Zavarzin, 1961). Attempts were made to isolate and purify this unusual organism from oligotrophic and eutrophic freshwater bodies, but with little success as it appeared only to grow in the presence of other microorganisms, particularly



Fig. 2.61 Metallogenium, as described by Zavarzin (1961).

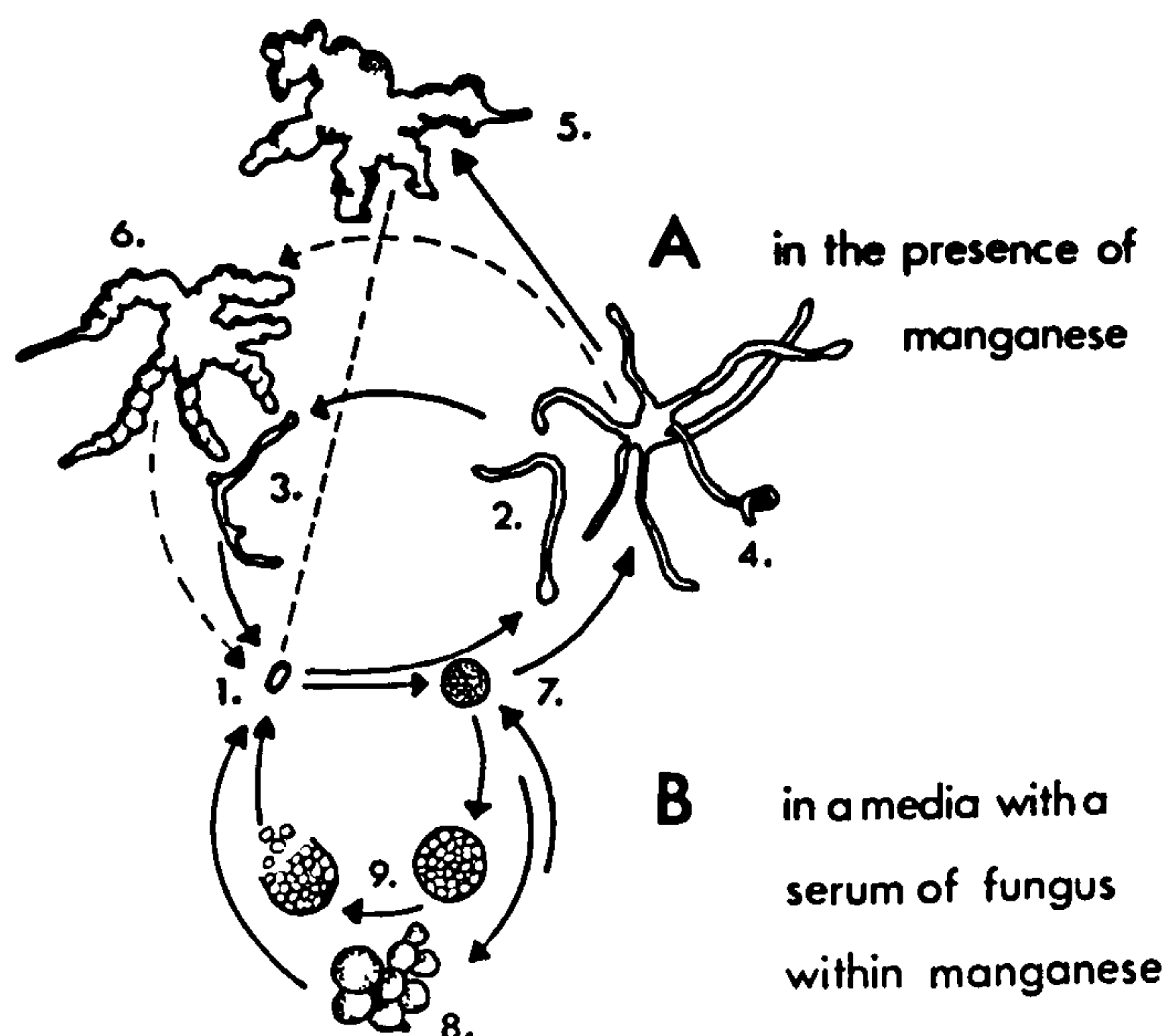


Fig. 2.62 Developmental cycle of Metallogenium, as described by Dubinina (1973). Cycle is composed of a unicellular motile stage (7, 8, 9 and 1), a multicellular stage with radial filaments (2, 3 and 4) and cell forms (5 and 6) encrusted in metal oxides.

the multicellular arrays of "Pedomicrobium" and other prosthecate bacteria which, after two years incubation, dominated the freshwater 'static' enrichments. These mixed cultures did, however, permit preliminary studies to be carried out which may reveal this organism as it is in nature, rather than by studying it artificially in pure culture.

Maintenance and culturing

A mixed culture containing Metallogenium was maintained in HB medium supplemented with 0.01% (w/v) manganous chloride, pH 7.0. Incubation was at 30° C.

Morphology

The morphology of this bacterium closely resembled the description of Dubinina (1973), being characterised by irregular filaments radiating from a common centre (Figs. 2.62, 2.64). The structure of the organism when first observed was unclear due to deposits, presumably metal oxides covering the cell body and filaments (Fig. 2.62c); however, in mixed cultures the deposits were considerably reduced (Fig. 2.65). The cells are coccoid with one to several filaments tapering at the tip. Filament branching was not observed; however, the filaments were frequently surrounded by a coat of manganese oxide (Fig. 2.64). The morphology of Metallogenium appeared to be affected by the presence or absence of manganese, and by the population of microorganisms naturally associated with it (Fig. 2.63).

Metallogenium - a valid genus ?

The microbial oxidation of manganese was studied by Tyler and Marshall (1967b) in hydroelectric pipelines. They showed that stalked

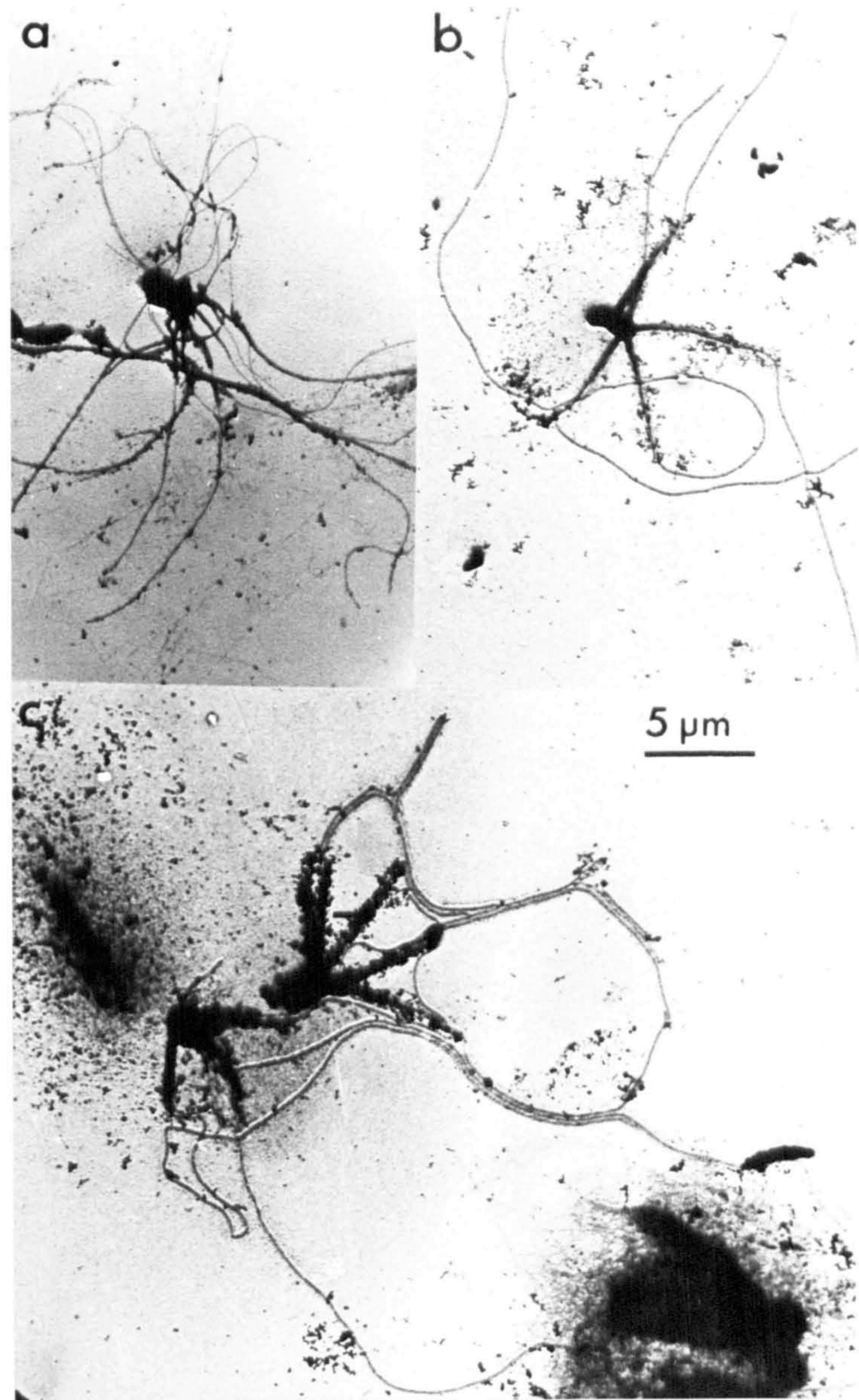


Fig. 2.63 Metallogenium, growing in manganese salts medium. Cell body and the 'radial filaments' were encased in metal deposits.

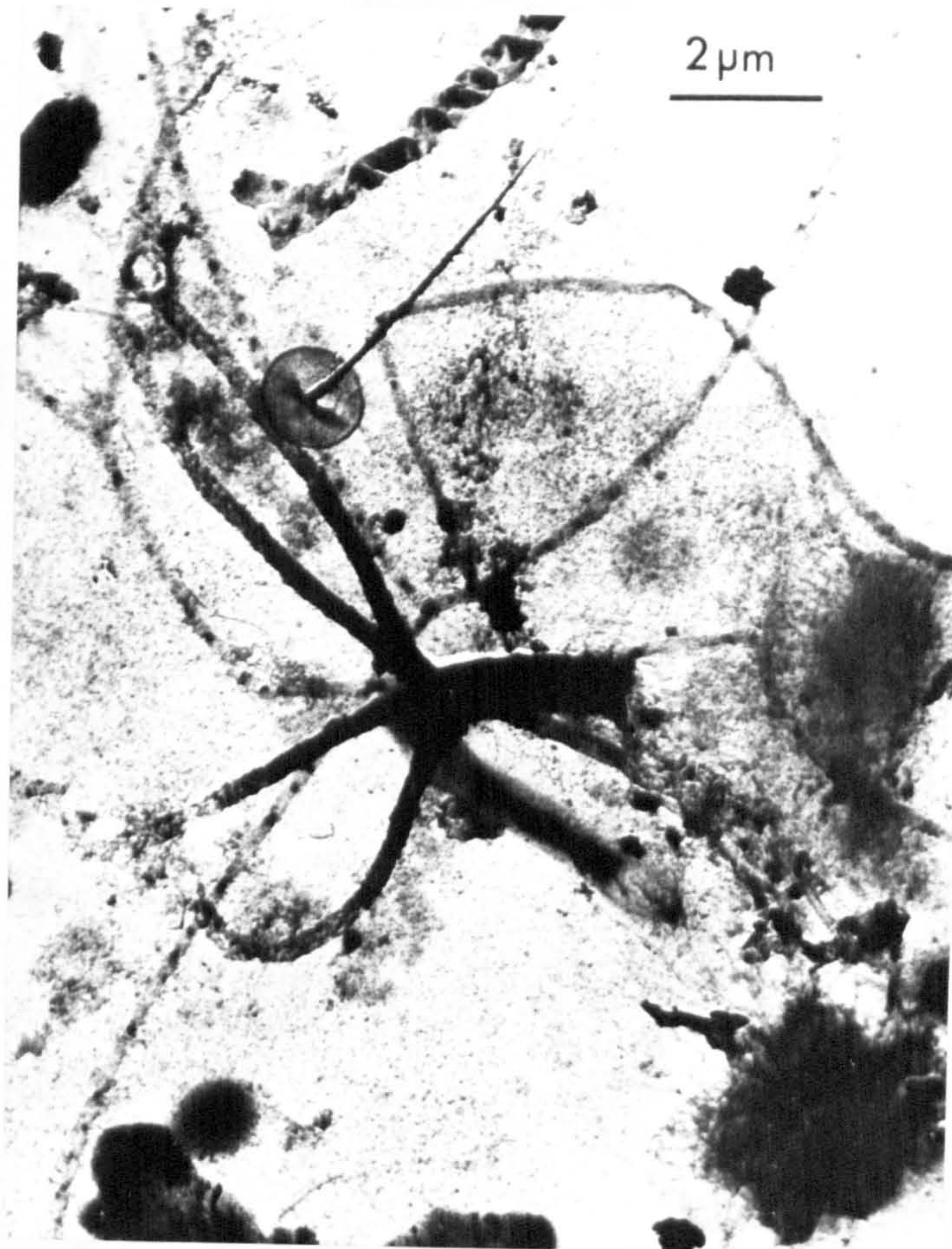


Fig. 2.64 Metallogenium, observed in 'static' enrichments, together with Seliberia and umbrella-shaped structures.

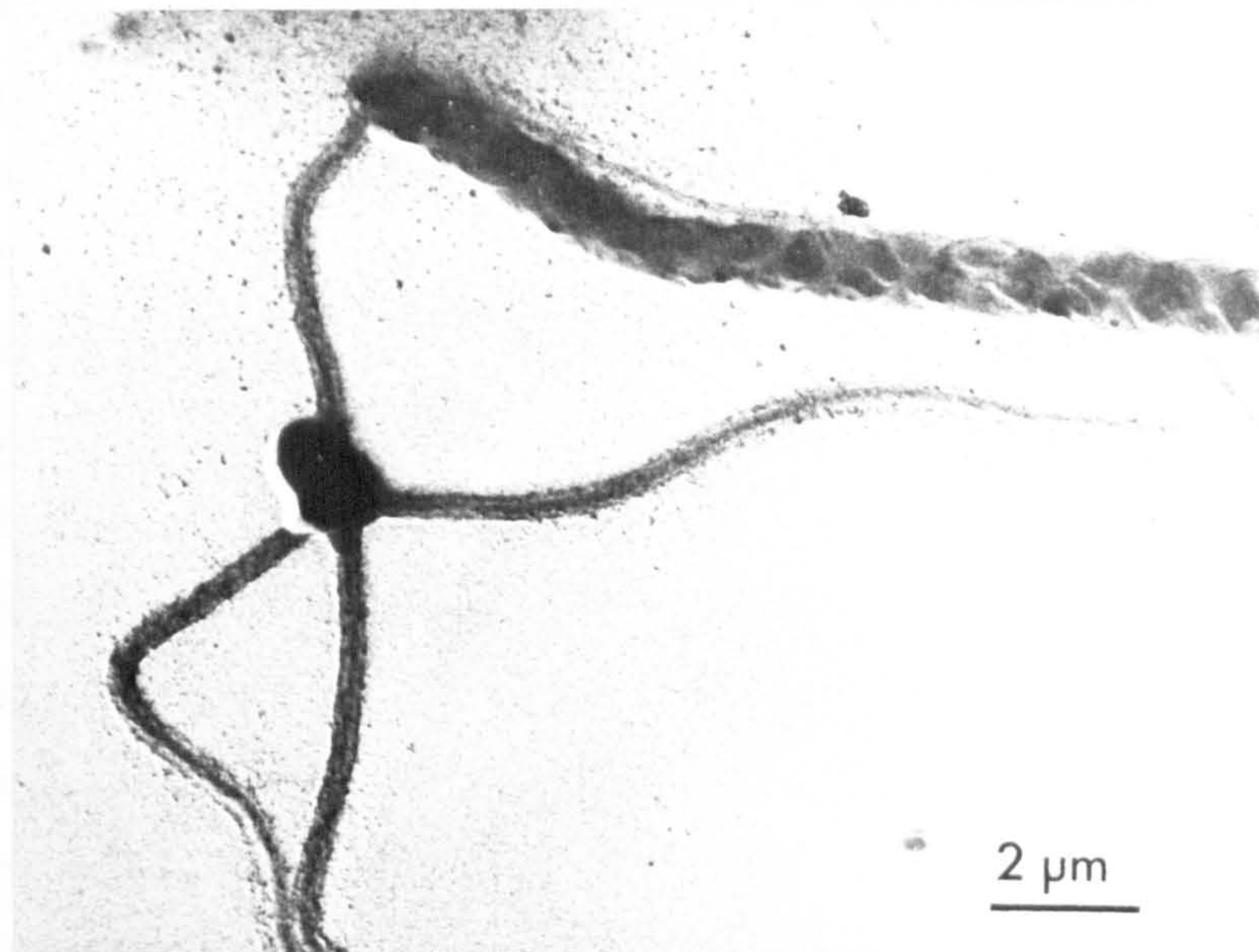


Fig. 2.65 Metallogenium, cell types in 'static' enrichments, showing the close resemblance to Pedomicrobium, i.e. appendages extending from the cell body.

budding bacteria (hyphomicrobia) were responsible for the manganese deposition (Section 3.III.4). They observed many bizarre forms of manganese oxidising hyphomicrobia, resembling Pedomicrobium as described by Aristovskya (1961), and Metallogenium symbioticum (Zavarzin, 1964, 1968). After studies on the pleomorphic nature of Hyphomicrobium, it was concluded that Pedomicrobium was "but one facies" of Hyphomicrobium (Tyler, 1970).

Observations made in this thesis, together with those of previous workers (Dubinina, 1973) question the taxonomic position of Metallogenium. Its ability to oxidise manganese in the natural environment, together with its budding mode of reproduction from filamentous outgrowth, closely relates this organism to Hyphomicrobium. The tendency of Metallogenium to grow only in the presence of other bacteria or fungus, suggesting symbiotic development, may be a consequence of an oligotrophic environment. Dubinina (1973) has shown that high concentrations of manganese can be substituted for the fungal symbiosis, to support a modified life cycle (Fig. 2.62), not too dissimilar from Hyphomicrobium T37 encrusted in manganese deposits (Tyler and Marshall, 1967).

(e) Planctomyces

Introduction

In 1924, Gimesi described a new colonial microorganism which formed rosettes, with numerous filiform appendages radiating from a common centre, the distal tip of each structure terminating in a spherical structure. He described this as a planktonic fungus, and named it Planctomyces bekefii. Henrici and Johnson (1935) found similar organisms attached to glass slides submerged in a lake, and described them as stalked, budding bacteria, to be named Blastocaulis sphaerica (Fig. 2.66). This organism has also been described as Gallionella kljasmiensis (Razumov, 1949), because the stalks were encrusted in iron, however this was later shown to be similar to Blastocaulis sphaerica (Zavarzin, 1961), and transferred to the genus Blastocaulis. Skuja (1964) has described another species,

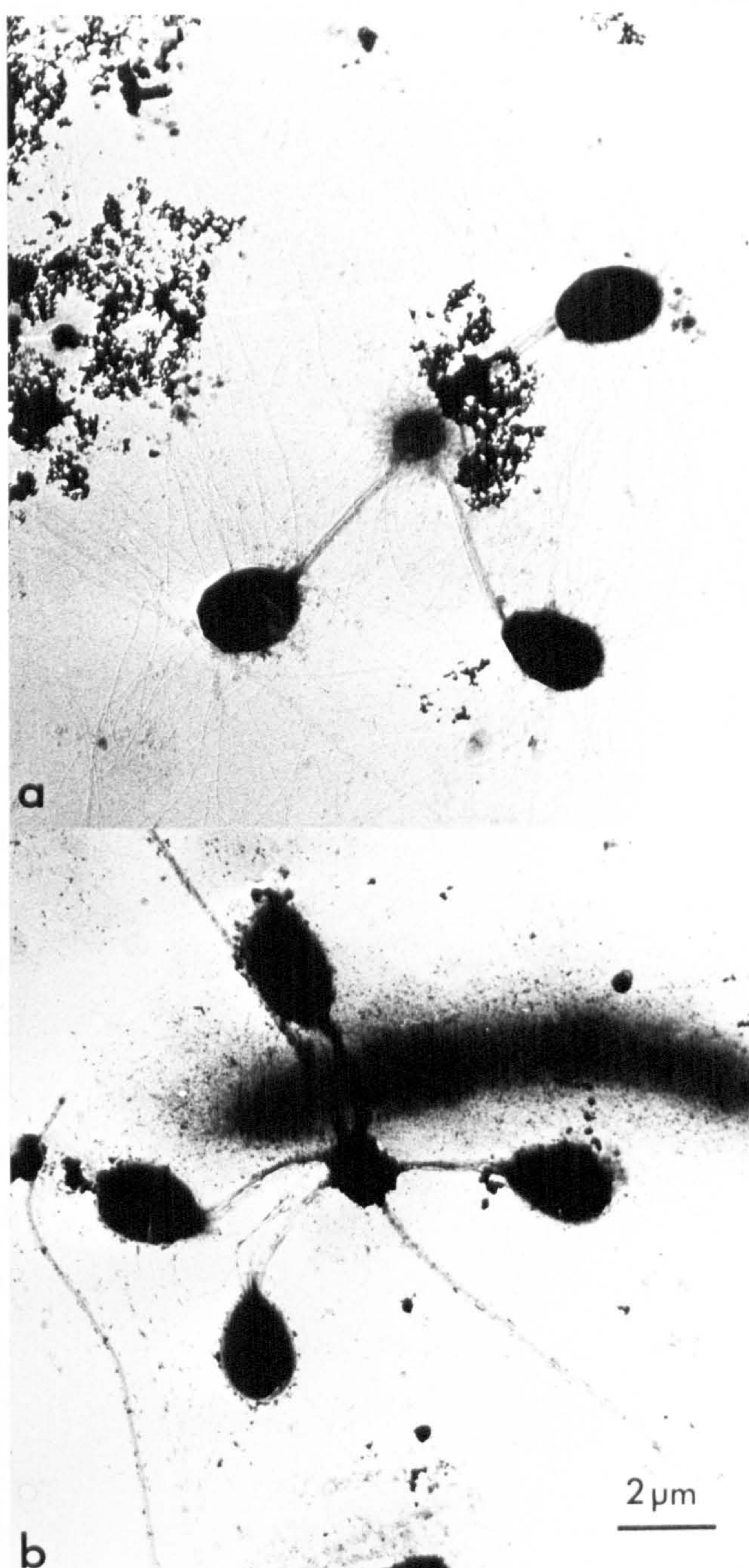


Fig. 2.66 Aggregates of Planctomyces ; cells aggregate about metal deposits, by means of holdfasts, to form rosettes. Note extensive arrays of fimbriae radiating from the cell bodies (a) and the fibrillar nature of the stalk. (Gold/Palladium shadowed).

P. condensatus, which differs from the others in that the stalk is short or absent, and can bear a terminal bud (cf. Staley, 1973). The cells are aggregated about a central iron containing material. This species closely resembles Blastobacter henrici as described by Henrici and Johnson (1935) and Zavarzin (1961). Among many other proposed species, only P. gracilis (Hortobagyi, 1965) has been considered valid; this species does not, however, appear to deposit iron material. None of these have been obtained in pure culture.

Kalbe et al. (1965) studied the morphology of Planctomyces under the electron microscope, after acid treatment to break down iron deposits. They demonstrated that the stalk was composed of fibres, and the length and width of the stalk varied considerably, although this could have been a result of the acid treatment. Numerous 'filaments' radiating from the cell body were also noted, but their function was not commented on. Oláh and Hajdu (1973) studied a population of P. bekefii which was only weakly encrusted in iron, thereby avoiding any acid treatment. They showed that the stalk was composed of several fibres running parallel, whose diameter was similar to that of the fibres radiating from the cell body.

These studies showed that Planctomyces appeared to be widely distributed and usually occurred during the late summer and early autumn in lakes and ponds (Hirsch, 1972). Oláh and Hajdu (1973) suggested that their recent mass occurrence may well be due to the increased eutrophication of water, but could also be due to unsuitable methods of detection being used in the past.

Bauld and Staley (1975) have been the only workers successful in obtaining a pure culture of a Planctomyces sp. This was a marine isolate, which resembled Planctomyces sp. previously observed in stored seawater and marine sediments (Hirsch and Rheinheimer, 1968). The cells were described as spherical to ovoid in shape, and the appendage, which was composed of several fibres, extended from one of poles. The appendage was not bound by any of the cell envelope layers, and thus it is a stalk and not a prostheca (Staley, 1968). A holdfast,

located at the tip of the stalk, enables the bacterium to attach to various surfaces. Fimbriae are located about the entire surface of the organism. Like many budding bacteria, mature cells of this organism exhibit only longitudinal symmetry. Bauld and Staley (1975) showed by slide culture and electron microscopy that reproduction was by budding only, the buds, which are motile by a single flagellum, being formed from the site of a protuberance at the free pole.

Results and Discussion

Freshwater isolate of a *Planctomyces* sp.

Bauld and Staley (1975) have shown that *Planctomyces* is indigenous to the marine environment from which it was isolated. It was proposed to isolate *Planctomyces* from freshwater environments, where it was frequently observed (Section 2.III.1). Waters undergoing early eutrophication, e.g. Draycote Water Reservoir, Esthwaite in the Lake District (sample site 10), when observed under the electron microscope showed a high incidence of this organism, relative to the prosthecate bacteria, and so enrichments were set up (Section 2.II). Dark brown colonies, growing deep into GMB agar, were composed of cells aggregated together. Appendages were not readily discernible when wet mounts of cultures were viewed by phase contrast microscopy, but electron microscopy identified their fibrillar nature.

Morphology

The Gram negative cells were round to ovoid in shape, tapering slightly at one pole where the appendage was located (Fig. 2.67). The cell diameter along the longitudinal axis was 1.5 - 2.0 μm . The stalk length was 2.5 - 3.0 μm and its diameter was 0.1 - 0.3 μm and ended in a holdfast. Many fibrils made up the bulk of the stalk, which was not bounded by any of the cell wall layers. Each fibre was approximately 50 Å thick, and the fibres were aligned parallel to one another along the length of the appendage. Shadowing and staining techniques were employed to try and elucidate the fine structure of the stalk. The apical tip was often encrusted in material resembling a type of metal complex, however on occasions these were absent and then the holdfast structure could be observed (Fig. 2.68). This

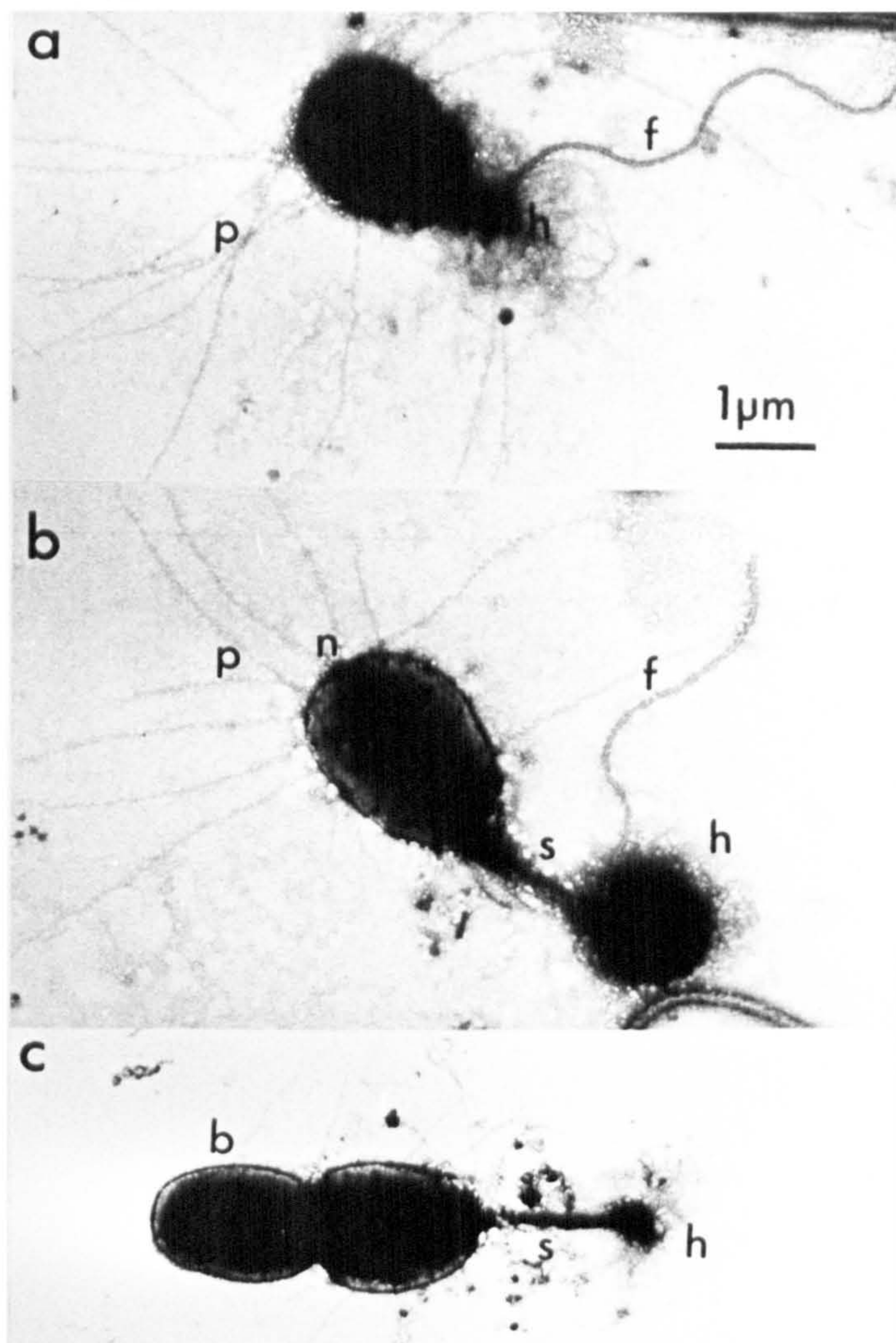


Fig. 2.67 Life cycle of Planctomyces. (a) Bud, having been constricted from mother cell develops holdfast (h), flagellum (f) and fimbriae (p). (b) Stalk grows out from the cell body with holdfast and flagellum at its apex. (c) Bud develops from nipple (n) to give rise to new cell. (Negatively stained).

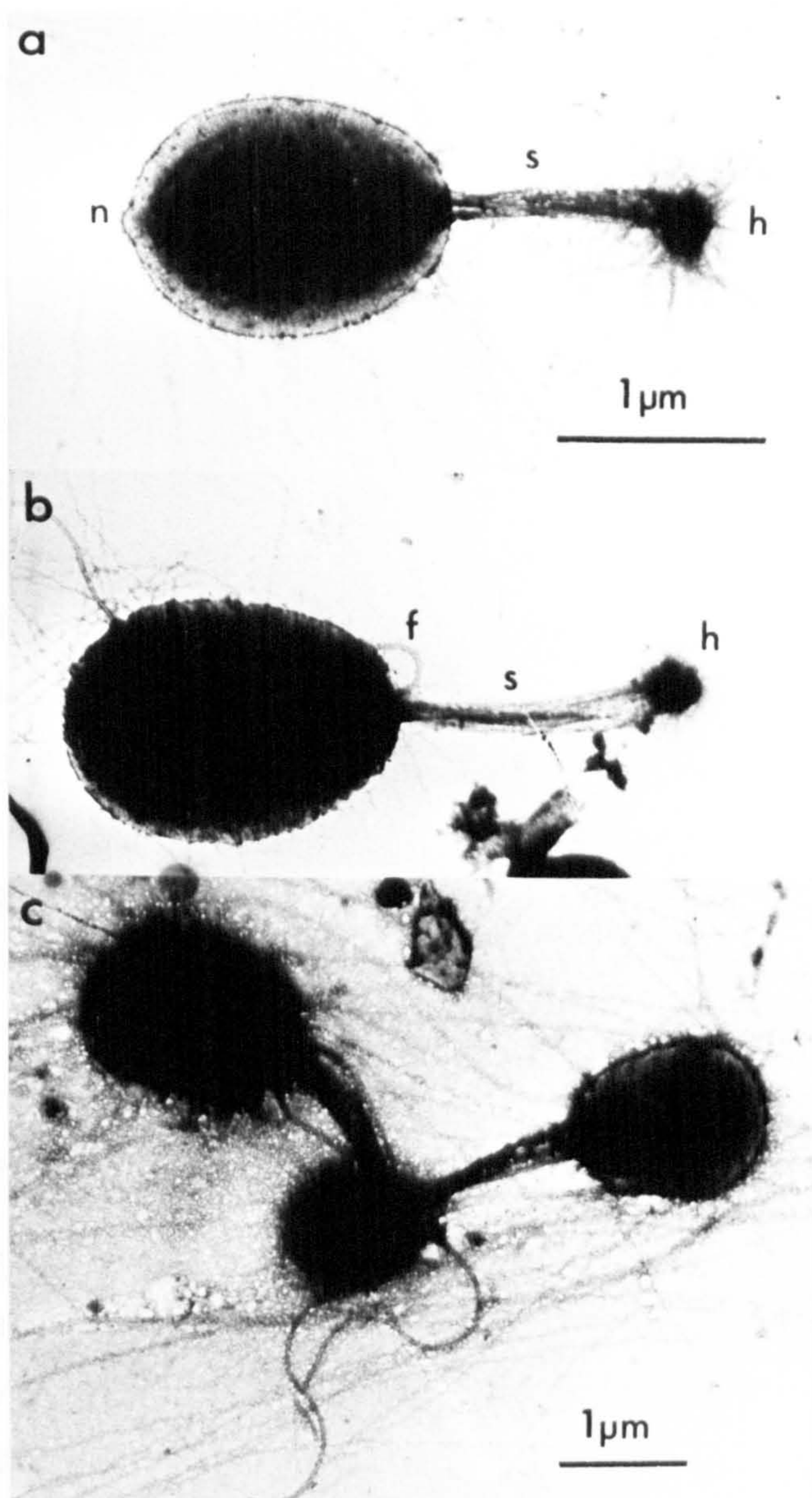


Fig. 2.68 Detailed micrographs of *Planctomyces*.

- (a) Mature cell showing characteristic protuberance or nipple (n), and developed holdfast (h) at the tip of the fibrillar stalk (s).
- (b) Flagellum (f) in subpolar position, is not shed throughout development cycle.
- (c) Flagellum (f) at tip of stalk. Fimbriae develop extensively from the cell bodies. (Negative shadowed).

structure appeared to be a network of fine fibres, binding the ends of the stalk fibres together (Fig. 2.68b). The basal region of the stalk was observed with great difficulty. The fibrils of the stalk appeared to emerge from several pores within the cell wall, but no defined structure could be observed. (Sectioning of the cells would have shown the fine structure of the stalk emanating from the polar region, however as growth in liquid culture met with little success, ultrastructural studies were not possible. No fine structure of Planctomyces has been published to date).

The sheathed flagellum was located in the subpolar position or at the tip of the stalk and did not appear to be shed ; its length varied from 3-5 μm . Numerous fimbriae extended over the entire surface of the cell (Fig. 2.69). Fimbriation is common amongst bacteria, however fimbriae reaching the lengths found with Planctomyces is unusual, and might be a reflection of the environmental conditions of growth (Section 2.III.1). It was not unusual for the length of fimbriae to be in excess of 30 μm . A small lump was detected at the non-stalked pole of the cell (Fig. 2.68a) in agreement with observations made by Bauld et al. (1975) who proposed that this protuberance, which marked the reproductive pole, represented the initial stages of bud formation.

Reproduction and life cycle

The life cycle was determined by slide culture studies and electron microscopy. Slide cultures developed slowly, but they showed that the mode of reproduction was budding, confirming the studies of Bauld et al. (1975). The bud arose from the site of the protuberance, by outgrowth, and upon reaching a certain size, the motile daughter cell was released by constriction from the mother cell (Fig. 2.70). It would appear from electron microscope studies that the bud, once released, undergoes a maturation period, although unlike many budding bacteria, this does not involve loss of motility through the shedding of flagellum. The fimbriae develop over the cell surface, a holdfast is formed, and finally the stalk structure develops.

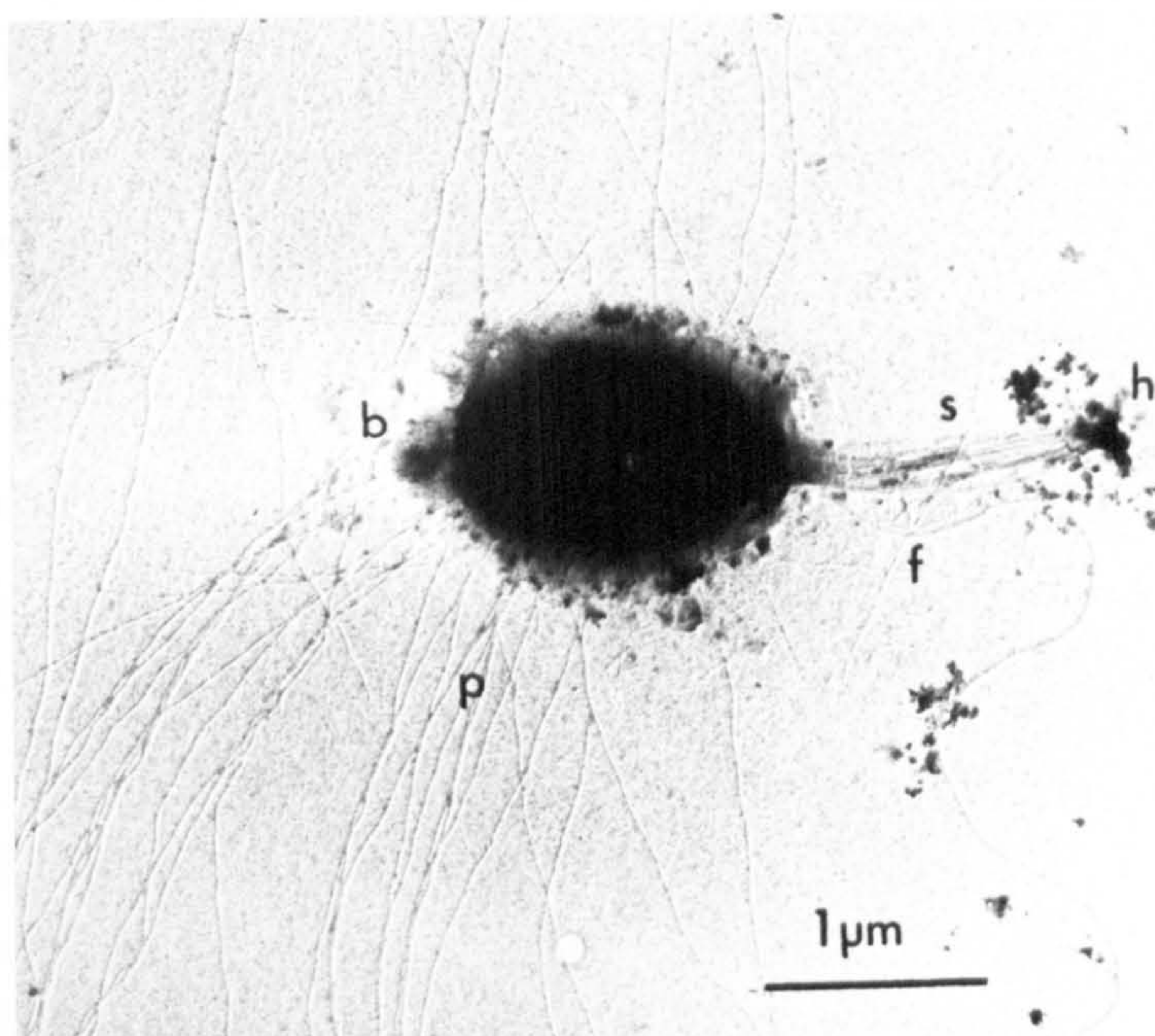


Fig. 2.69 Mature cell, illustrating the extensive display of fimbriae radiating from the cell body. The fibrillar nature of the stalk is evident. The bud is starting to develop (b). (Gold/Palladium shadowed).

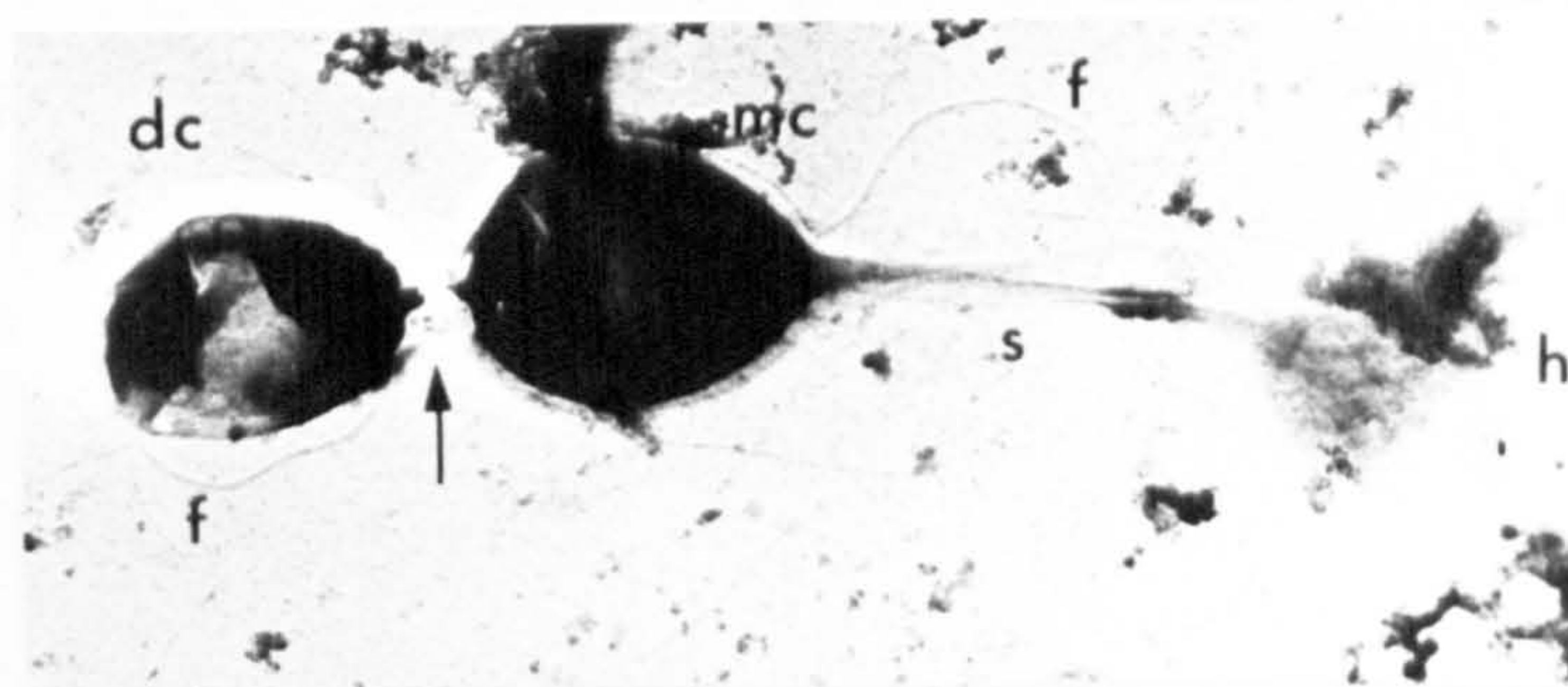


Fig. 2.70 Daughter cell about to constrict from the mother cell, possesses a flagellum, but must develop holdfast, fimbriae and stalk to repeat the life cycle again. (Gold/Palladium shadowed).

Like many of the prosthecate bacteria, Planctomyces possesses a dimorphic life cycle, with the cell going through a time ordered sequence of events which culminates in the production of a daughter cell. In this respect the life cycle of Planctomyces is analogous to that found in Caulobacter (Shapiro, 1971) and Asticcacaulis (Pate and Ordal, 1965) where stalk development is obligate to the life cycle, but is not involved in the reproductive process. In Caulobacter and Asticcacaulis, the appendage is a true cellular extension or prostheca (Staley, 1968), however similarities between the life cycles of Caulobacter and Planctomyces do challenge any significant role that the stalk of Caulobacter might play in reproduction (Section 2.III.2(a)), especially as studies on Caulobacter (Fukuda et al., 1977) and Asticcacaulis biprosthecum (Pate et al., 1973) have shown that these bacteria can grow and divide in the absence of their stalks.

This isolate of Planctomyces closely resembled the marine isolate morphologically, however it did not grow in any salinity range. In addition, the stalk of this freshwater isolate appeared shorter and thicker than previously described species (Buchanan and Gibbons, 1974). Apart from the stalk, the freshwater isolate resembled P. bekefii; the differences in stalk dimensions could be attributed to culture conditions.

Before this isolate can be properly characterised, there must be improved growth in liquid culture, to enable studies to be carried out on fine structure, nutrition, physiology and for its (G + C) base ratio to be determined.

Planctomyces in the natural environment

Bacterial surveys of freshwater bodies showed that under conditions of low nutrient concentrations, Planctomyces sp. maintained reasonable numbers in the total population, frequently being observed in association with prosthecate bacteria (Section 2.III.1). The holdfast structure was clearly in evidence, enabling these bacteria to adhere to other cells or inert substrata (Figs. 2.71, 2.72). The pili network also became extensive in the nutrient poor environment. Species of Planctomyces



Fig. 2.71 Planctomyces sp. in the natural environment, showing well developed holdfast structures.

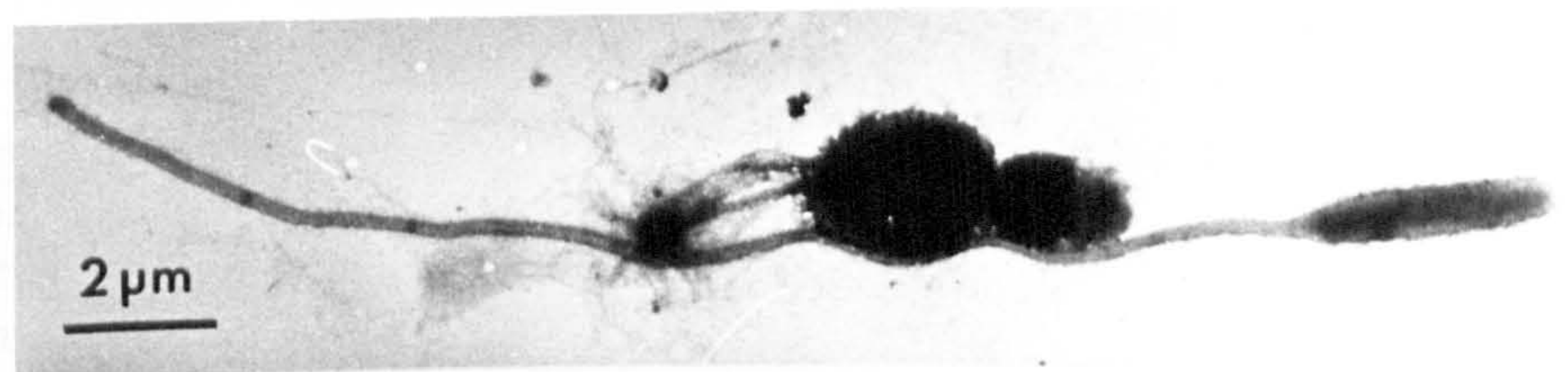


Fig. 2.72 Planctomyces sp. and a Caulobacter sp. in the natural environment.

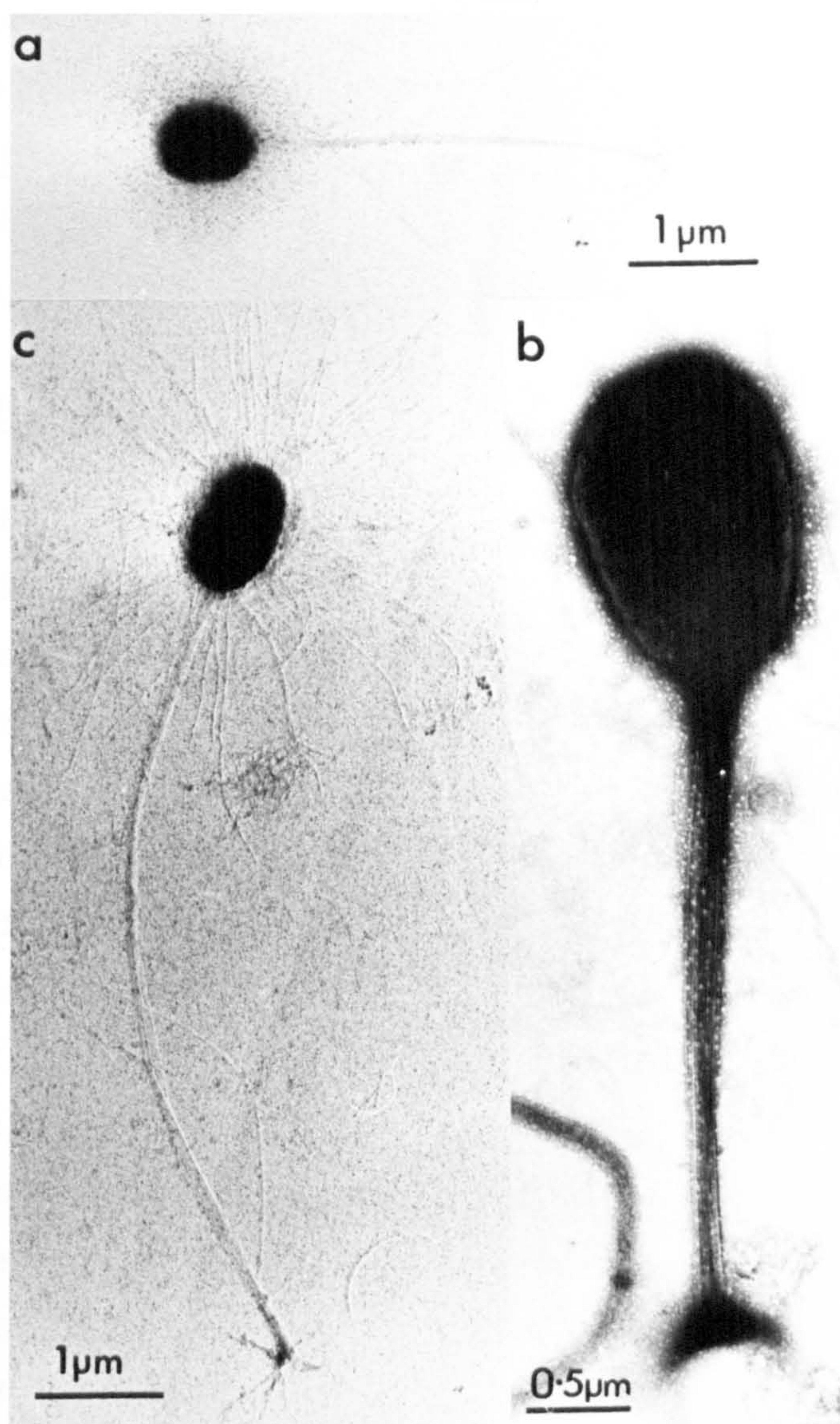


Fig. 2.73 *Planctomyces* sp. observed in oligotrophic waters (Haweswater) possessed varied morphologies from isolates observed in eutrophic waters (e.g. Estwaite and Draycote Reservoir in early autumn).

- (a) species lacking fimbriae or developed holdfast structure.
- (b) species lacking fimbriae and holdfast.
- (c) species possessing radial fimbriae on cell body and about the holdfast structure.

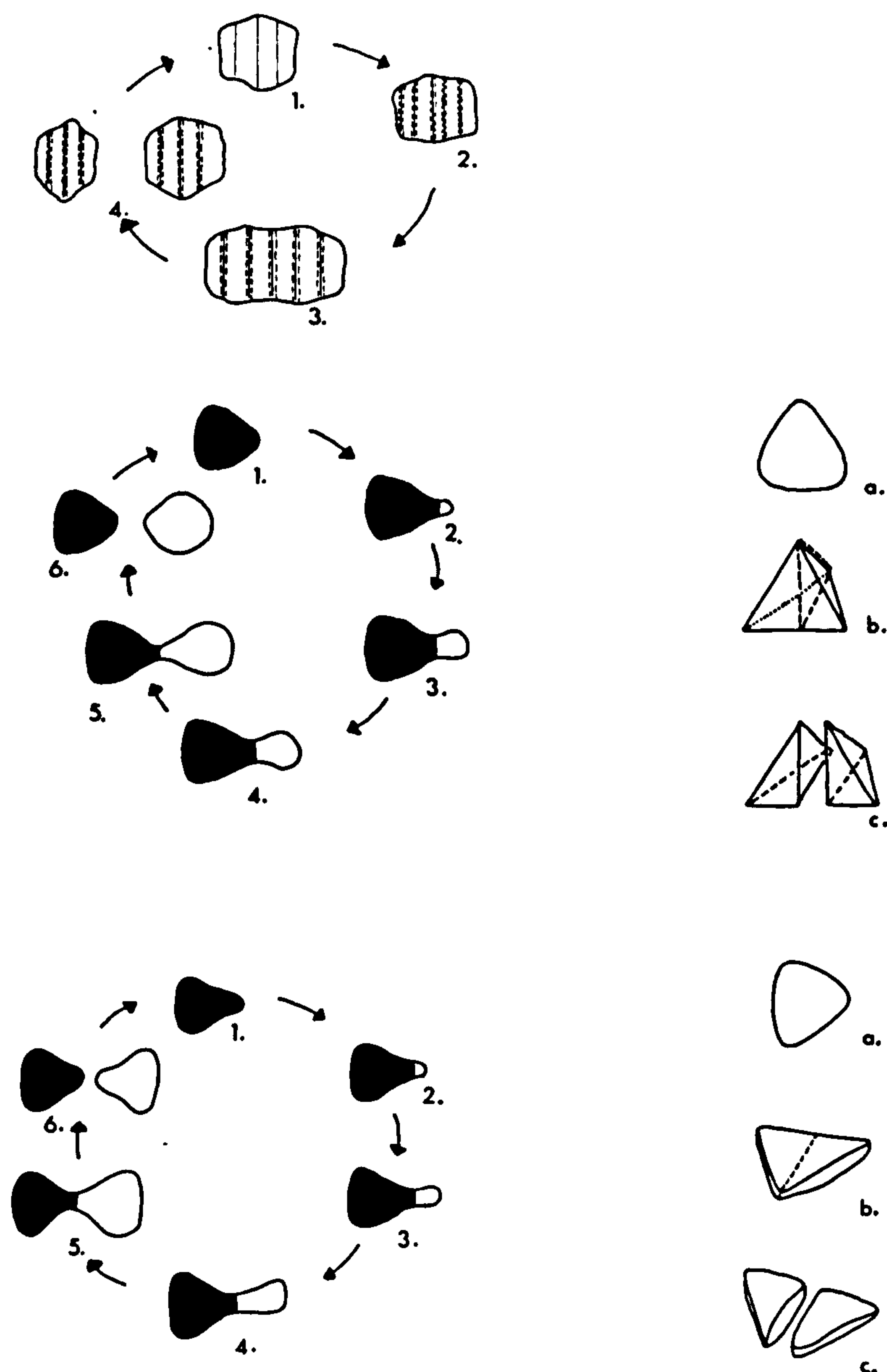
[(a) and (b) negatively stained; (c) gold/palladium shadowed]

lacking pili (Fig. 2.73) were present when the water was originally monitored, but they rapidly disappeared as the samples were maintained without any nutrient replacement. Planctomyces clearly represents an organism which has adapted to its environment, the stalk enables the cell to draw nutrients from a greater surrounding volume of water (cf. Caulobacter); however, as the acellular stalk is not an integral part of the cell, it cannot serve as an uptake organelle. The fimbriae may well compensate for this by concentrating molecules in the environment.

(f) The mushroom-shaped bacteria

Introduction

Whittenbury and Nicoll (1971) isolated a Gram negative mushroom-shaped bacterium from pond water which multiplied by a budding process. In 1972 Namsaraev and Zavarzin isolated a morphologically similar organism from lowland marsh which was given the name 'tetrahedron'; this isolate also reproduced by budding from one vertex of the tetrahedron. Both isolates were nonmotile, and formed a mucous capsule. Lafitskaya and Vasil'eva (1976) isolated a bacterium which they proposed to be different from the two earlier isolates, as it had the appearance of a flat triangle with the elongation of one of the vertices of the triangle, to give rise to a bud, which eventually gained the size and shape of the mother cell. A new bud on the daughter cell, as in the case of the mother cell, was formed at the site of detachment, thus the cell retained the same polarity. The bacterium isolated by Lafitskaya and Vasil'eva (1976), strain 1109, is described as having radial symmetry, a property common to Stella humosa (Vasil'eva et al., 1974) (Fig. 2.74); however, Stella humosa reproduces by binary fission rather than by budding. Stanley, Moore and Staley (1976) isolated two new strains of mushroom-shaped bacteria, which were morphologically similar to previous isolates from England and Russia. However, their DNA/DNA homology studies indicated their strains and those from other workers not to be identical to one another.

**Fig. 2.74**

Proposed life cycles of (a) *Stella humosa*, (b) mushroom-shaped bacterium and *Tetrahedron*, (c) triangle-shaped bacterium, strain 1109. Sketches on right depict three-dimensional structures of these bacteria, as described in the text.

Results and Discussion

During enrichment studies for budding and prosthecate bacteria, a mushroom-shaped organism was observed, and enriched for on PWPY and later GMB medium (Section 2.II).

Freshwater isolate of a mushroom-shaped bacterium

Triangle-shaped bacteria were observed in water samples collected from Draycote Water Reservoir (Fig. 2.75). Their unusual morphology characterised them as being similar to mushroom-shaped budding bacteria isolated previously. These bacteria were enriched for, and round, white, slightly mucoid colonies gave rise to pure cultures of this unusual Gram negative bacterium.

Physiology

This isolate, designated MpD, only grew aerobically; growth under nitrogen with 0.2% (w/v) nitrate was negligible. Of the carbon compounds tested, MpD utilised formate, lactate, propionate, acetate, citrate, glucose, galactose and glutamate as carbon and energy source. Methanol and methylamine were poorly utilised.

Growth occurred between 12°-37° C, the optimum growth rate being at 30° C, at which temperature it grew optimally between pH 6.0 and 8.0.

Base composition

The DNA of MpD had a buoyant density of 1.7251 g/cc³ and contained 66.5 moles % guanine plus cytosine (G + C) to total bases.

Growth cycle and morphology

This isolate appeared very similar in morphology to previous isolates of mushroom-shaped bacteria, as revealed by light and electron microscopy (Figs. 2.76, 2.78). Culturing of this isolate caused its shape to become rounded, so that it closely resembled the isolate of Whittenbury and Nicoll (1971). Slide culture studies confirmed the budding mode of multiplication, with a generation time of 4 h (Fig. 2.77). Newly divided organisms were rounded on one

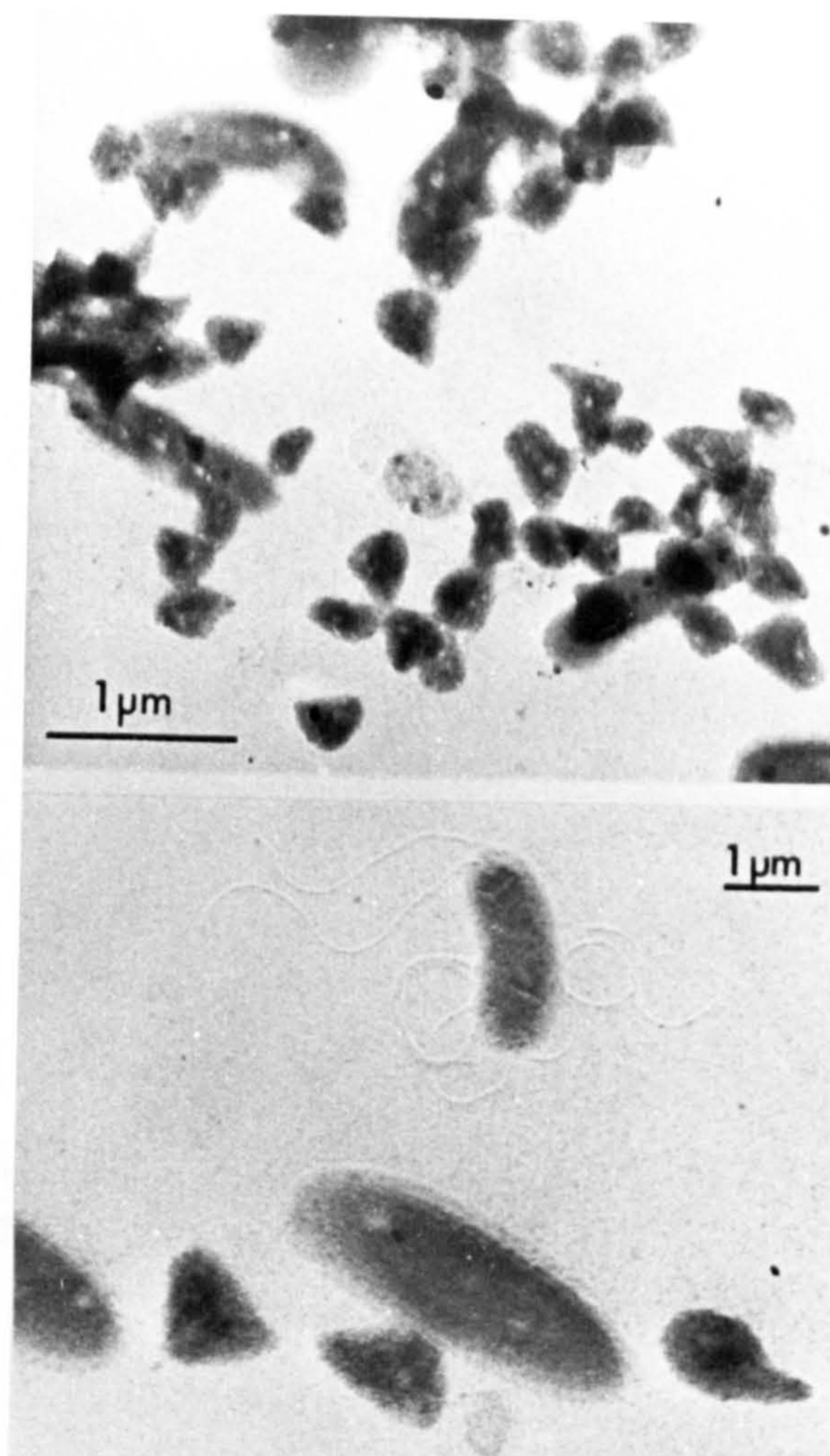


Fig. 2.75 Triangle-shaped bacteria observed in water samples collected from Draycote Reservoir.

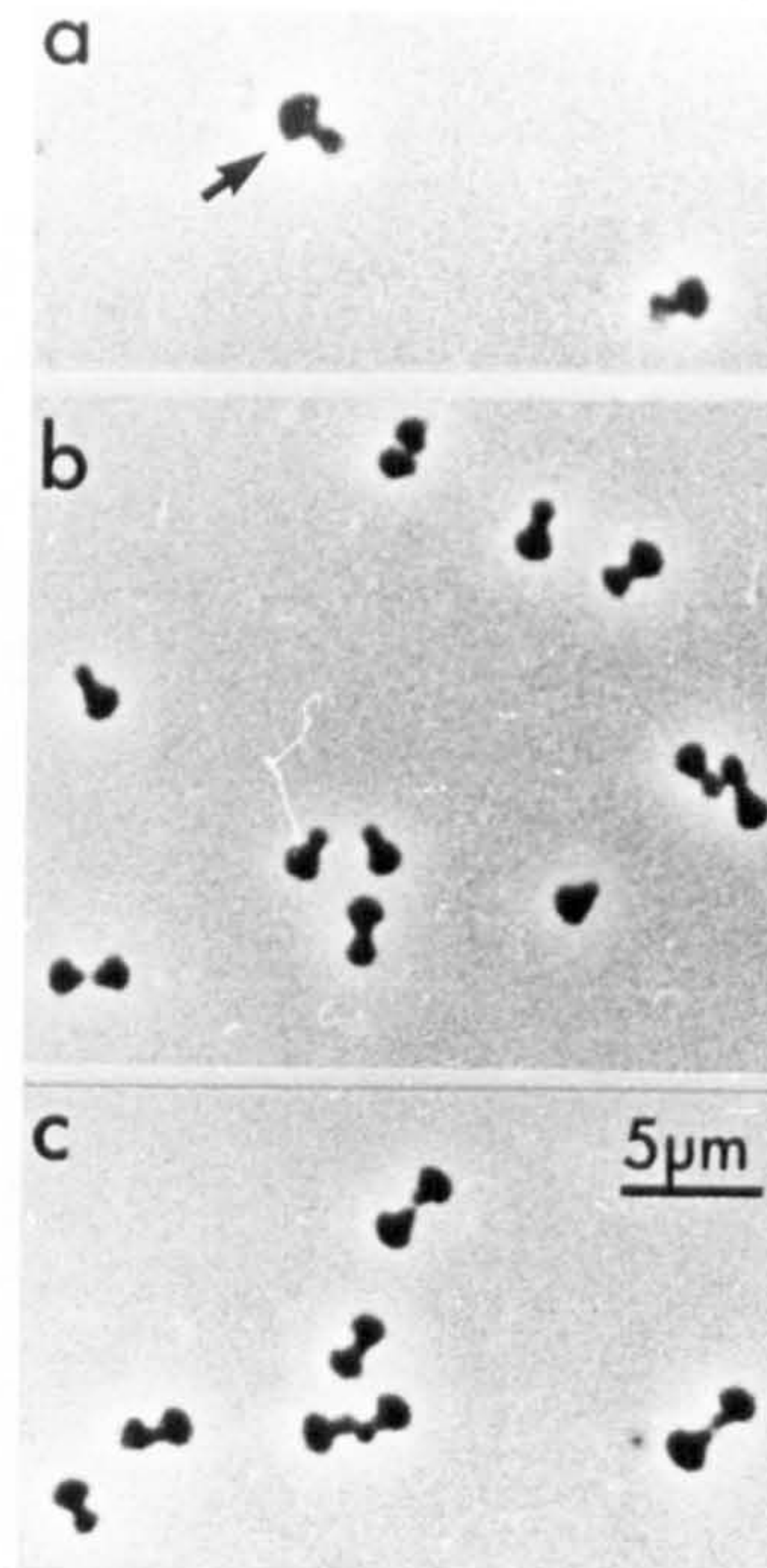


Fig. 2.76 Pure isolate of mushroom-shaped bacterium. Light micrographs illustrate its unusual morphology.

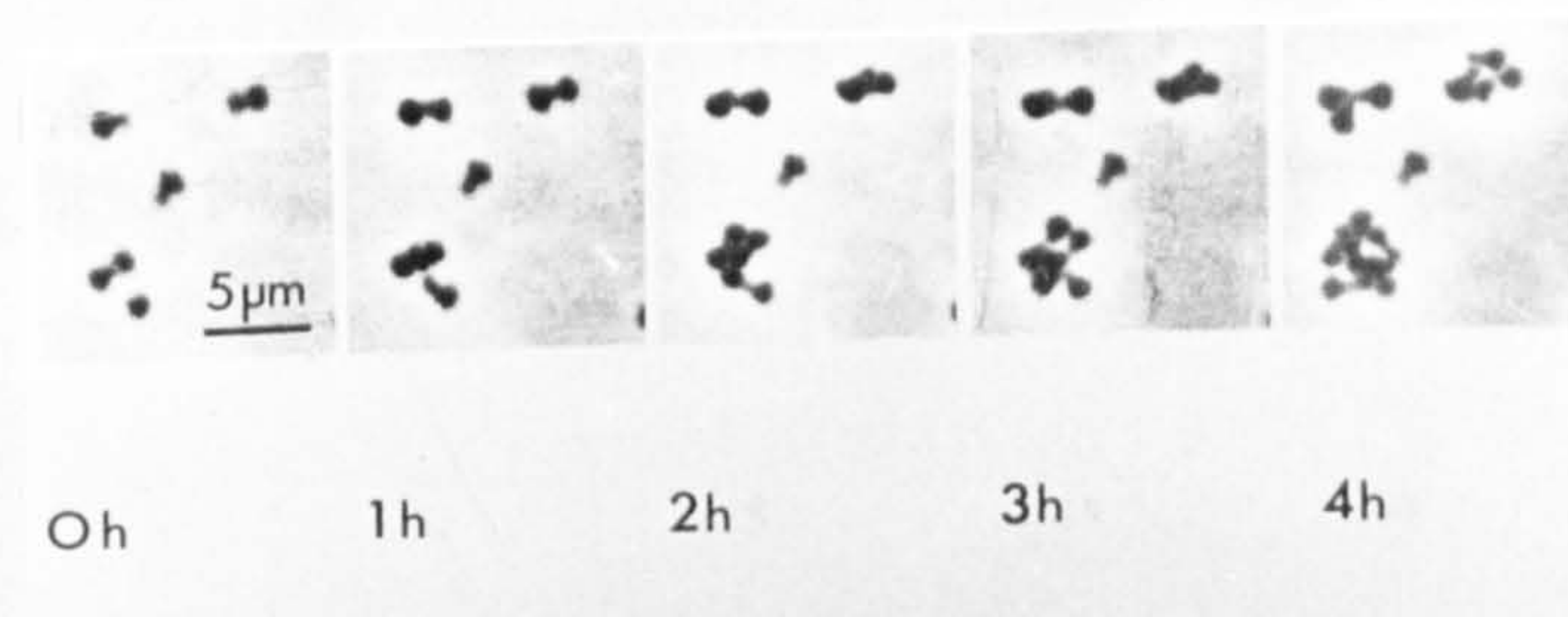


Fig. 2.77 Slide culture of the mushroom-shaped bacterium, showing its budding mode of reproduction. Mother and daughter cells are equivalent at division. 2nd generation buds form from previous plane of division.

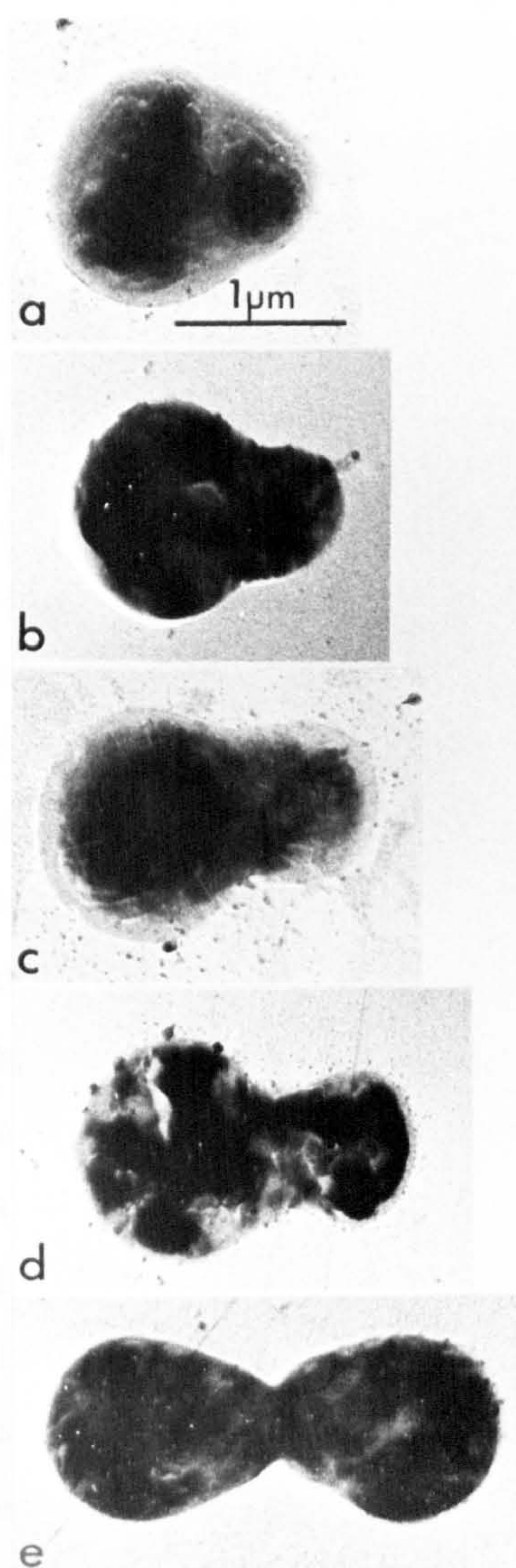


Fig. 2.78 Electron micrographs of the mushroom-shaped bacterium through the life cycle, indicated by slide culture studies. Although reproduction is by asymmetric polar growth (budding); at division mother and daughter cells are equivalent (e).

side, whereas the vertex where cell separation had occurred appeared conical, giving rise to the characteristic mushroom-shape (Fig. 2.76a). Electron microscope studies showed that this organism was nonmotile and did not possess a holdfast structure. The conical region of the cell elongated to form a tube which then swelled and enlarged to give rise to a dumb-bell shaped organism. Prior to binary division, the mother and daughter cells were of equal size. The cells divided by constriction to give mother and daughter cells of equivalent size, which then produced buds at the point of separation. Although this budding process is analogous to that described for other tubed, budding bacteria (Whittenbury and McLee, 1967; Staley, 1968; Whittenbury and Dow, 1977), division in this case is symmetric, giving rise to two equivalent cells. The budding organisms Pasteuria ramosa (Staley, 1973) and Nitrobacter winogradsky (Murray and Watson, 1965), although without a characteristic cellular extension, have the presence of a flagellum in immature cells, and in the case of Pasteuria ramosa a holdfast structure in mature cells (Fig. 2.79), to distinguish the mother and daughter cells. The mushroom-shaped bacteria, together with a bacterium isolated from the intestine Gemmlinger formicilus (Gossling and Moore, 1975) (Fig. 2.79) appear to be the only budding bacteria that do not go through an obligate maturation stage, and consequently have a monomorphic life cycle.

In agreement with the studies carried out by Whittenbury and Nicoll (1971), phosphate deficiency was shown to have a negligible effect upon 'tube' length of the isolate MpD, unlike many other budding prosthecate bacteria with 'true' appendages (Schmidt and Stanier, 1966), although it did cause an increase in generation time from 4 h with 0.1% (w/v) phosphate salts to 15 h with 0.001% (w/v) phosphate salts.

Fine structure

Ultrastructural studies of mushroom-shaped bacteria showed that there did not appear to be a complex membraneous system, common to many budding bacteria (Fig. 2.80). Some degree of compartmentalisation was observed, however, suggesting the presence of some transverse membranes (Fig. 2.80).

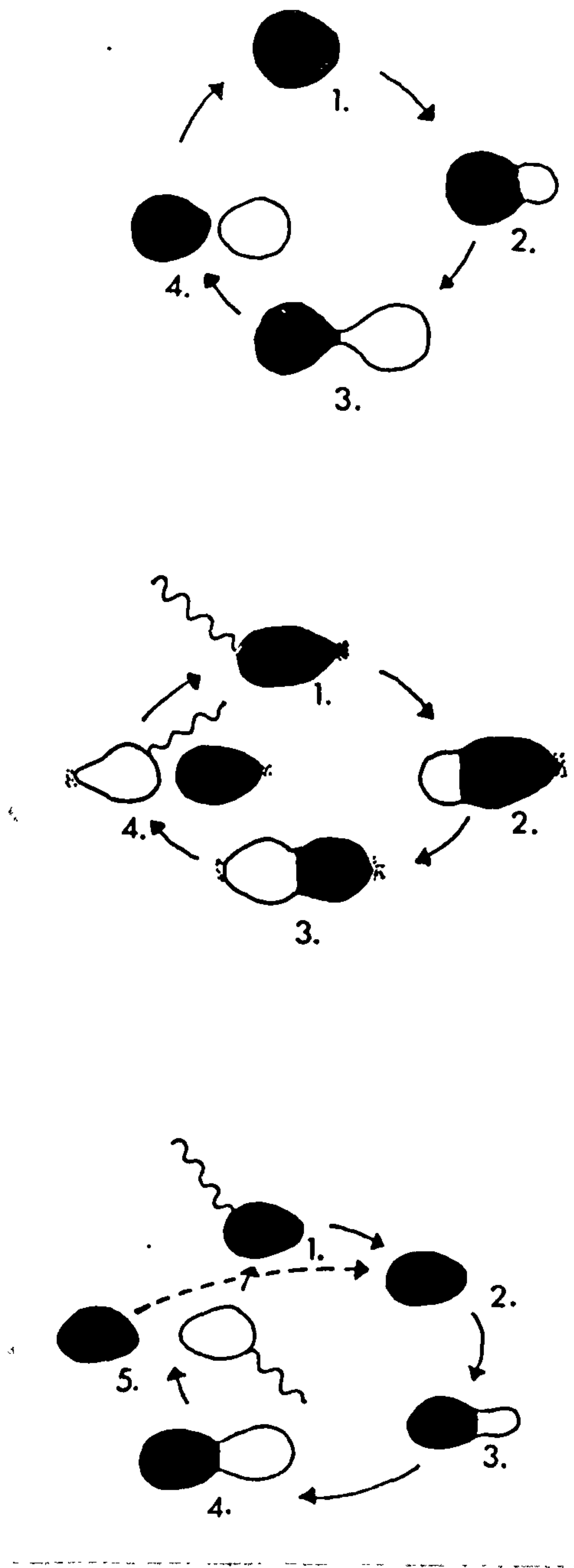


Fig. 2.79 Proposed life cycles of

- (a) Gemminger formillicus ,
- (b) Pasteuria ramosa (Blastobacter, Staley (1973)),
- (c) Nitrobacter winogradsky.

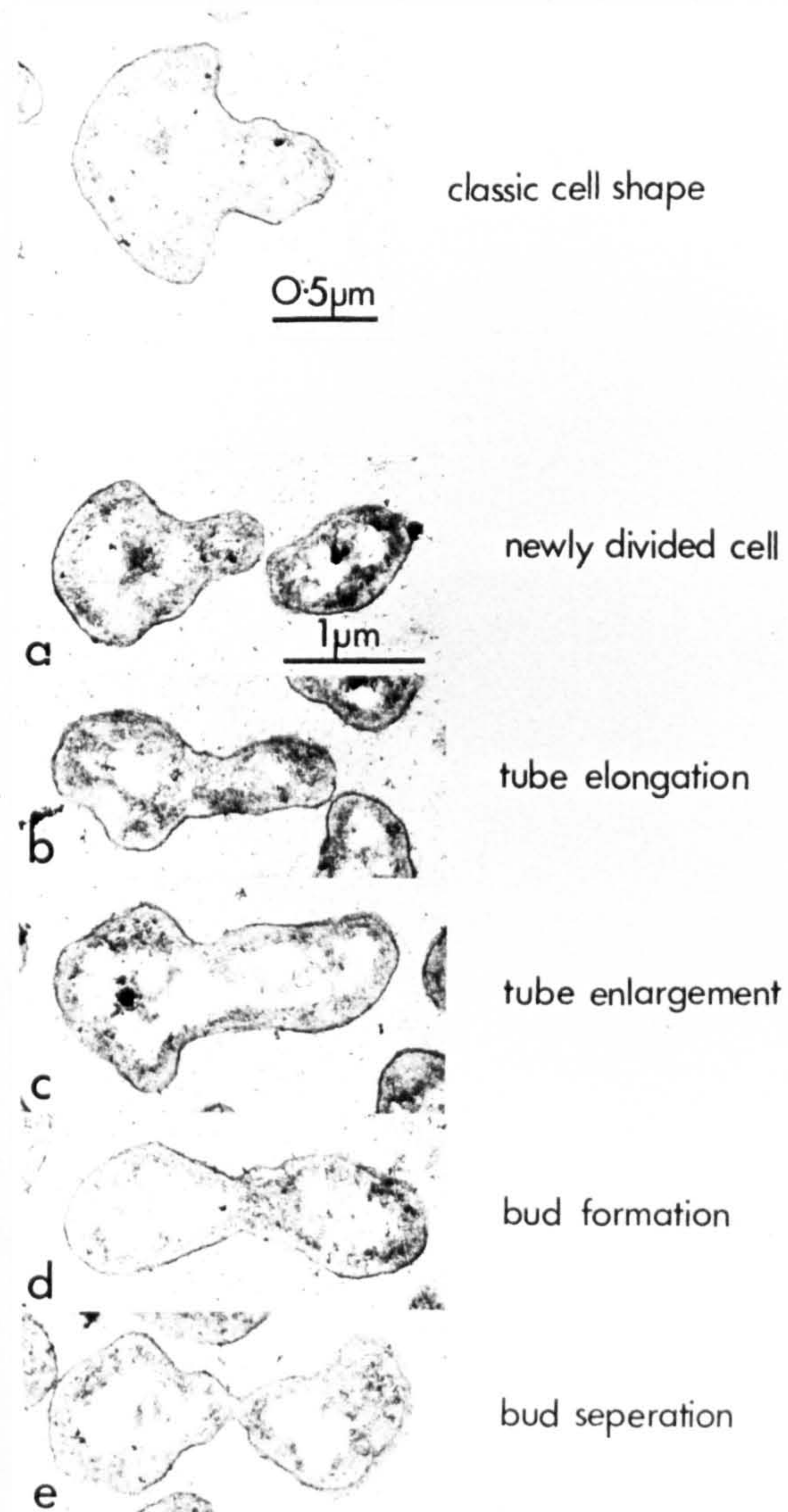


Fig. 2.80 Ultrathin sections of the mushroom-shaped bacterial isolate. Longitudinal sections through the cells demonstrated the absence of complex membranes, although some degree of compartmentalisation was evident. (Light area represents nuclear material).

This study has shown that this isolate of a mushroom-shaped budding bacterium is very similar to previous isolates with respect to morphology and physiology. As yet no genus has been proposed for these morphologically unusual bacteria (Whittenbury, personal communication).

Section 3

A. Hyphomicrobium. A study on a budding prosthecate bacterium.

I. Introduction

Hyphomicrobium is a prosthecate bacterium which reproduces by a budding process (Figs. 3.1 & 3.2). Multiplication occurs by the formation of a bud or daughter cell at the tip of the stalk. During its maturation the bud develops a polar flagellum, and after a period of active movement, the bud 'breaks off' from the stalk of the mother cell to become a motile swarmer cell. The swarmer cell then undergoes a period of maturation, when it loses its flagellum and increases in size, and eventually starts to develop a stalk from one of its poles. This stalked cell can then develop a bud, to repeat the life cycle. Meanwhile the mother cell, after a short period during which there is further stalk growth, develops a new bud. As the two cells are not equivalent, the life cycle of Hyphomicrobium is dimorphic, composed of a daughter cell cycle and a mother cell cycle. The dimorphic cell cycle is common to most of the budding prosthecate bacteria, i.e. Caulobacter, Planctomyces and Rhodomicrobium, as previously described (Section 1).

Hyphomicrobium was originally observed in enrichment cultures for nitrifying bacteria. (Rullman, 1897; Stutzer and Hartleb, 1898; Fred. and Davenport, 1921; Prouty, 1928), and in 1936 Kingma-Boltjes succeeded in isolating Hyphomicrobium vulgare from such an enrichment culture. He identified the cellular extension of this organism as a stalk and described the mode of reproduction as budding, and so Hyphomicrobium lost its earlier associations with fungi (Stutzer and Hartleb, 1898). Subsequently Henrici and Johnson (1935) grouped this organism with other stalked bacteria; however, Stanier and Van Niel (1941) considered that as the mode of reproduction was budding, these organisms could not be included in the Schizomycetes, but must be placed apart in a provisional appendix. Since that time Hyphomicrobium has been studied extensively (Mevius, 1953; Näveke, 1957; Zavarzin, 1960; Guillard and Watson, 1962; Leifson, 1964;

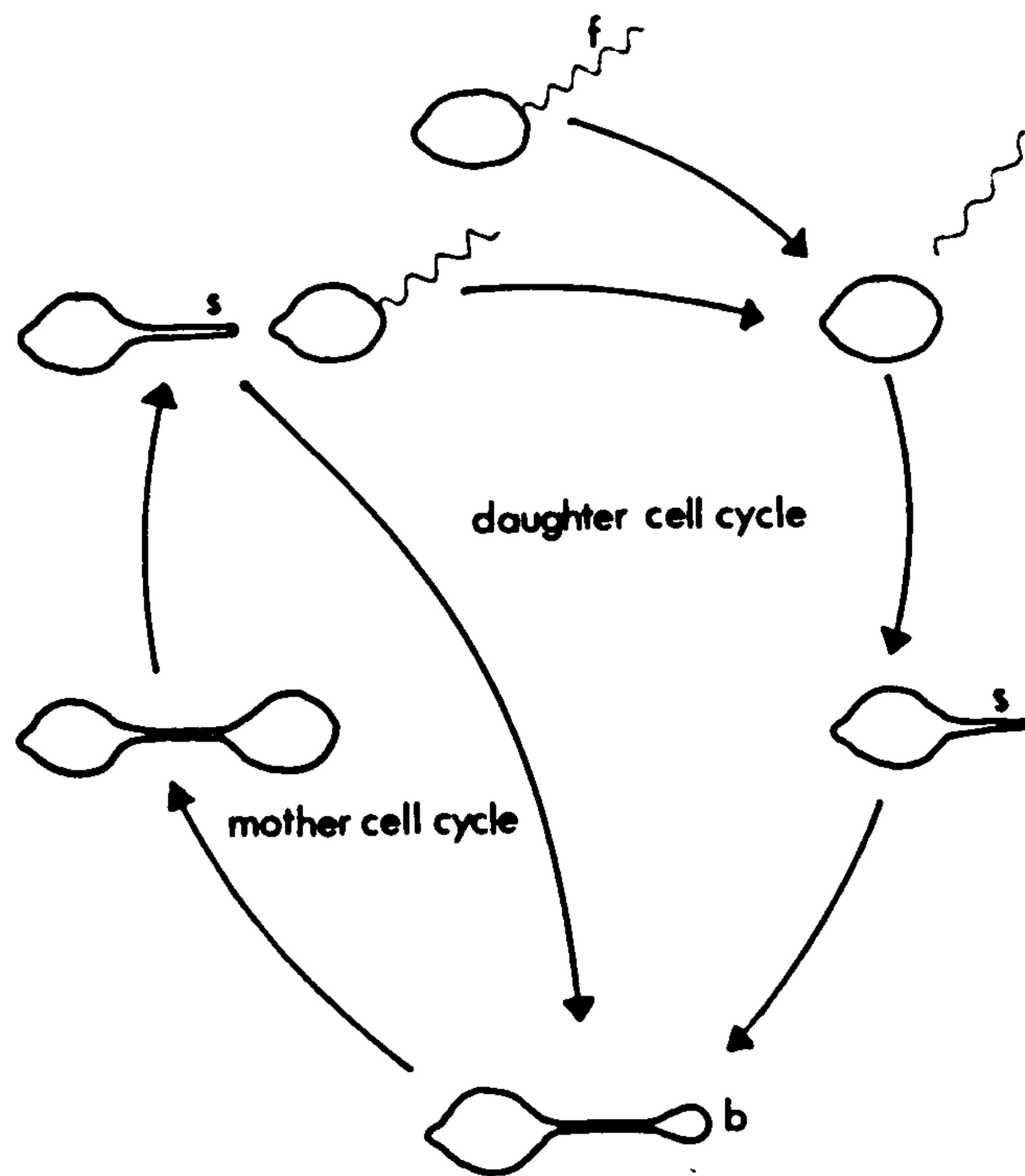


Fig. 3.1 Life cycle of *Hyphomicrobium* sp. Swarmer cell matures, shedding flagellum (f), develops a stalk (s) and finally a bud (b). The bud eventually constricts from mother to repeat the daughter cell cycle; the stalked cell develops a new bud, to continue the mother cell cycle, to give a dimorphic life cycle.

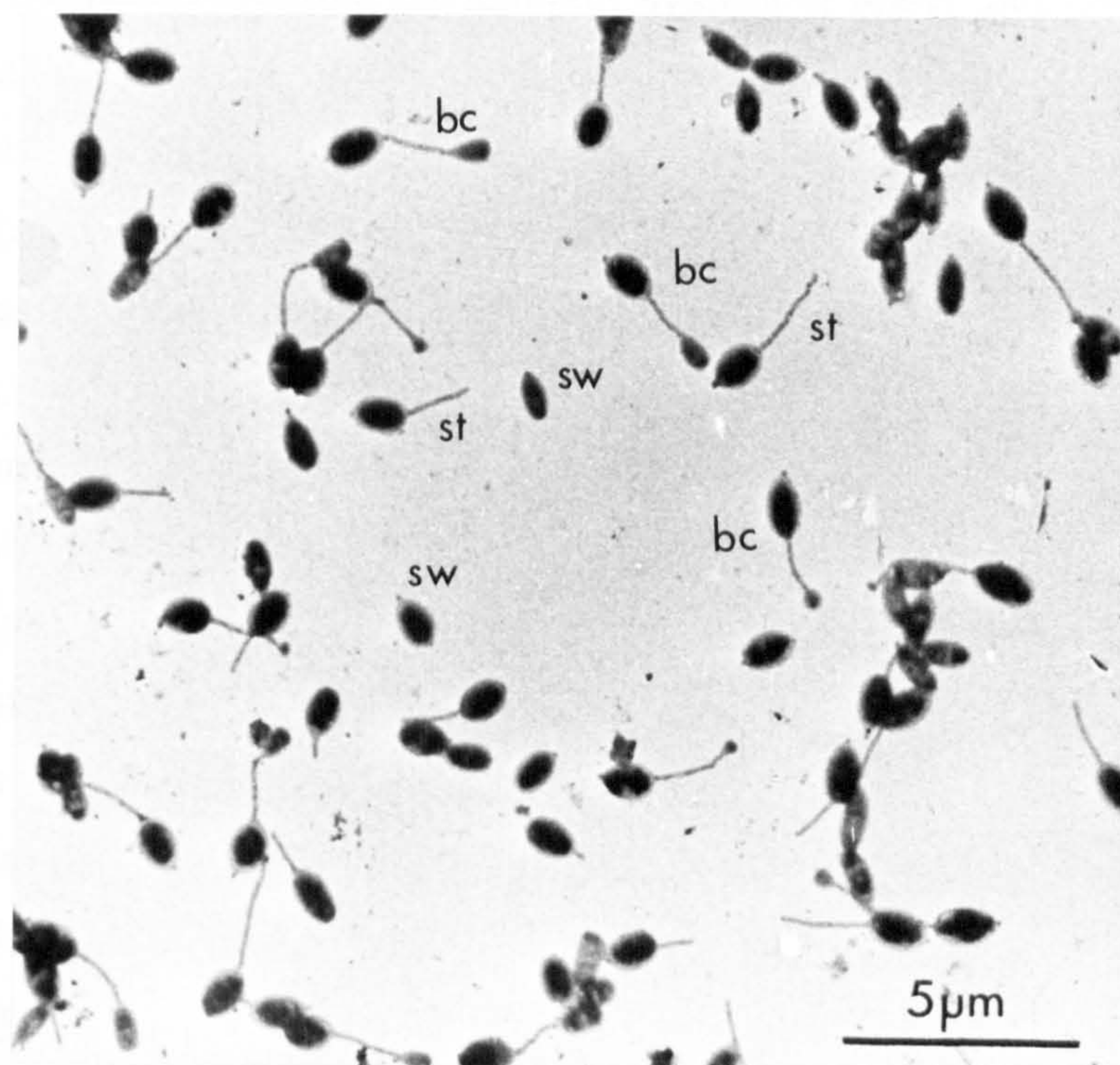


Fig. 3.2 Heterogeneous population of *Hyphomicrobium* isolate, Hy. 1, showing various cell types, swarmer, stalked and budding cells. Gold/Palladium shadowed.

sw = swarmer

st = stalked cell

bc = budding cell

Hirsch and Conti, 1964; Geitler, 1965; Shishkina and Trotsenko, 1974; Hirsch, 1974; Takada, 1975; Attwood and Harder, 1972, 1973, 1974, 1977; Monosov and Kudinova, 1976). Much is now known about the biochemistry of Hyphomicrobium (Harder and Attwood, 1978), but comparatively little is known about stalk formation, the budding mode of reproduction, polymorphic life cycles and the role of Hyphomicrobium in the environment. Until recently, one problem was the lack of a selective enrichment procedure for the isolation of Hyphomicrobium; however, the denitrification enrichment system of Sperl and Hoare (1971) and Attwood and Harder (1972) has yielded isolates capable of growing at higher growth rates, with higher growth yields, than previous isolates. Also these new isolates grow with uniform turbidity and clumps of cells or cell rosettes are rarely observed (Zavarzin, 1960).

For any detailed study on growth and morphology of Hyphomicrobium, synchronised populations of cells are required. Throughout the synchronisation procedure there is an obvious necessity to disturb the cells physiologically as little as possible. Consequently, if an organism possesses novel or unique features, these have been exploited, e.g. the holdfast properties of Caulobacter cells (Degnen and Newton, 1972a; Staley and Jordan, 1973), the multicellular arrays and swarm cells of Rhodomicrobium (Whittenbury and Dow, 1977). With E. coli (B/r strain), however, it has been possible to obtain synchronised populations by physically separating the cells at a particular stage of growth (Helmstetter and Cummings, 1963; Mitchison and Vincent, 1965). One of the most promising of the synchronisation techniques is the use of differential gradient centrifugation which has been successfully employed with E. coli (Mitchison and Vincent, 1965), with Chlorella (Baldwin and Wegener, 1975), Caulobacter (Stove and Stanier, 1962; Poindexter, 1964; Shapiro et al., 1971; Retnasabapathy, personal communication) and Rhodopseudomonas palustris (Whittenbury and McLee, 1967; Westmacott and Primrose, 1976). Lloyd et al. (1975)

have also synchronised cultures successfully by continuous flow size selection. With Hyphomicrobium, Moore and Hirsch (1973) have obtained synchronised swarmer cell cultures by centrifugation and filtration. During the centrifugation step, cell clumps and mother-daughter cells are pelleted and the supernatant then filtered through nitrocellulose membrane filters (3 μm pore). This filtration method had been previously used by Bauld, Tyler and Marshall (1971). One disadvantage is that this method gives generation times of 14 hours with a 5 hour lag before stalk synthesis, clearly indicating that the cell population is physiologically disturbed. Brandon and Norris (1972) described a continuous flow method which they employed for H. vulgare, where mother cells with filaments were supported on glass beads by natural adhesion and swarmer cells were continually released. Blackman and Weiner (1973) proposed inducing cell synchronisation in Hyphomicrobium, by the addition of nalidixic acid. In Hyphomicrobium naladixic acid inhibits deoxyribonucleic acid synthesis, but not ribonucleic acid synthesis for a period up to 5 hours, the inhibitor being initially bacteriostatic, thereafter bacteriocidal. The inhibitor was used at a concentration of 100 $\mu\text{g/ml.}$, and blocked bud formation and bud separation. These workers proposed washing out the inhibitor after 5 hours to synchronise the cells. Monosov and Kudinova (1976) synchronised a Hyphomicrobium sp. with helical prostheca thermally; however, no details were given.

Induction synchronisation and the method of centrifugation and filtration were applied to cultures of Hyphomicrobium, however, synchronisation was poor, the cells took a long time to initiate growth and aberrant forms were frequently observed. As Hyphomicrobium generally does not possess any developed holdfast material (cf. Zavarzin, 1960), the method of Helmstetter and Cummings (1964) could not be employed. The size differential between swarmer cells and budding cells was too small to employ simple selective filtration (Brandon and Norris, 1972; Dow, 1974). Hyphomicrobium was synchronised

successfully by using a modification of the method of Mitchison and Vincent (1965). This gave consistently good synchrony with the minimum of physiological shock. Homogeneous swarm cell populations therefore serve as a starting point in studies of cellular morphogenesis and differentiation.

Recent reviews (Starr and Skerman, 1965; Schmidt, 1971; Hirsch, 1974) have proposed that budding bacteria are distinguished from other bacteria in that they have evolved a specialised system of polar growth, resulting in two asymmetric cells, whereas in cells like E. coli division usually results in two symmetrical siblings. As previously noted (section 1), Donachie and Begg (1970) have shown that E. coli can grow by asymmetric polar growth under certain growth conditions, although optimally growth is by intercalation. Budding bacteria are distinguished from E. coli in that uni-directional growth is the normal mode of growth and is obligate under all growth conditions. The stalks of Hyphomicrobium, like Rhodomicrobium, (Whittenbury and Dow, 1977) are intimately involved in daughter cell formation, that is they are obligate to the life cycle and contain nuclear and cytoplasmic material destined for the daughter cell. The synthesis and movement of DNA has been studied by previous workers (Zavarzin, 1960; Hirsch and Jones, 1968) prior to and during bud formation, but the results have not been conclusive. It seemed appropriate, therefore, to study Hyphomicrobium as synchronised and heterogeneous populations in order to clarify the life cycle of this pleomorphic bacterium.

Studies on Hyphomicrobium in the natural environment and in pure culture have shown that this bacterium is pleomorphic. Extensive studies have been carried out by Hirsch and Conti (1964), Leifson (1964) and Bauld, Tyler and Marshall (1967, 1971) complementing earlier work carried out by Zavarzin (1960). Up to now, the identification of Hyphomicrobium has been based largely on morphological descriptions (Bergey's Manual of Determinative Bacteriology, 8th Edition, 1974) which classifies it as a prosthecate bacterium, because it reproduces

by 'budding' (asymmetrical binary fission) and possesses a prostheca. Such a classical description of the morphology of Hyphomicrobium fails to accommodate many of the observations made on the pleomorphism of this organism. The following studies were undertaken, in order to ascertain whether the pleomorphic forms observed in Hyphomicrobium cultures were artefacts or should be included in the description of the genus. Previous workers agree that cell shape, length of hyphae (stalks) and degree of branching in Hyphomicrobium depend on cultural conditions; however, the life cycle as described (Fig. 3.1) may be considered to be an artefact in that in the environment Hyphomicrobium sps. seldom encounter nutrient concentrations similar to those of laboratory cultures, i.e. pleomorphism as described may in fact be the normal morphological expression in the natural environment. This statement is justified in the light of the following studies on pleomorphism, i.e. the nutrient status of the medium was varied, and the effects on morphology monitored.

Hyphomicrobium grows optimally on methanol and methylamine, and to some extent on formate and urea (Hirsch and Conti, 1964; Attwood and Harder, 1978). The pathway of carbon assimilation is thought to be the following. It is considered that there is a central pathway which consists of three successive oxidations, leading to the dissimilation of reduced one-carbon compounds via formaldehyde and formate to carbon dioxide (Fig. 2.7) (Harder, Attwood and Quayle, 1973; Harder and Attwood, 1978). The one-carbon units are then incorporated at the oxidation levels of both formaldehyde and carbon dioxide into the serine pathway (Large et al., 1961), whereby the net synthesis of a three-carbon skeleton from one-carbon compounds is accomplished (Fig. 3.3). Two reduced one-carbon units at the oxidation level of formaldehyde condense with two glycine molecules to form two molecules of serine, which are then converted through a series of reactions to phosphoenol pyruvate. At this point the third one-carbon unit required for the synthesis of a three-carbon unit is incorporated in the form of carbon dioxide to form oxaloacetate and

finally malate. Malate is then activated to malyl CoA which is then cleaved to glyoxylate and acetyl CoA. The glyoxylate is converted to glycine and re-enters the cycle. Bellion and Spain (1976) proposed the regeneration of glyoxylate through the oxidation of acetyl CoA to isocitrate which is cleaved by isocitrate lyase to glyoxalate and succinate, the latter for assimilation into cell constituents; however, Harder and Attwood (1978) failed to detect any measurable amount of isocitrate lyase activity in all the isolates they tested, and therefore favour the icl^- variant of the serine pathway (Attwood, 1977)(Fig. 3.3).

Studies by Hirsch and Conti (1964b) showed that glutamate, formamide, lactate, succinate and aspartate would also support growth of Hyphomicrobium, and Sperl and Hoare (1971) demonstrated growth of two strains of Hyphomicrobium on n-propanol, n-butanol, glycerol and succinate; however, the long incubation periods required in both these studies suggest that the substrates tested were not utilised, and that Hyphomicrobium was growing at the expense of volatile carbon compounds in the atmosphere (Kingma Boltjes, 1936).

The carbon source can have a marked effect on the morphology of the organism (Bauld, Tyler and Marshall, 1971), as can the presence of certain sources of fixed nitrogen (Hirsch and Conti, 1964) and the presence of metals, especially manganese and iron, both of which alter the cellular and colonial expression of Hyphomicrobium (Tyler and Marshall, 1967; Hirsch, 1974).

Prosthecae bacteria which reproduce by a budding process are placed in one of three genera: Hyphomicrobium, Hyphomonas or Pedomicrobium. Members of the genus Pedomicrobium produce prosthecae from several sites all over the cell body, whereas Hyphomicrobium and Hyphomonas produce prosthecae from only one or both poles of the cell. Hyphomonas is separated from Hyphomicrobium on the basis of DNA-DNA homology studies (Moore and

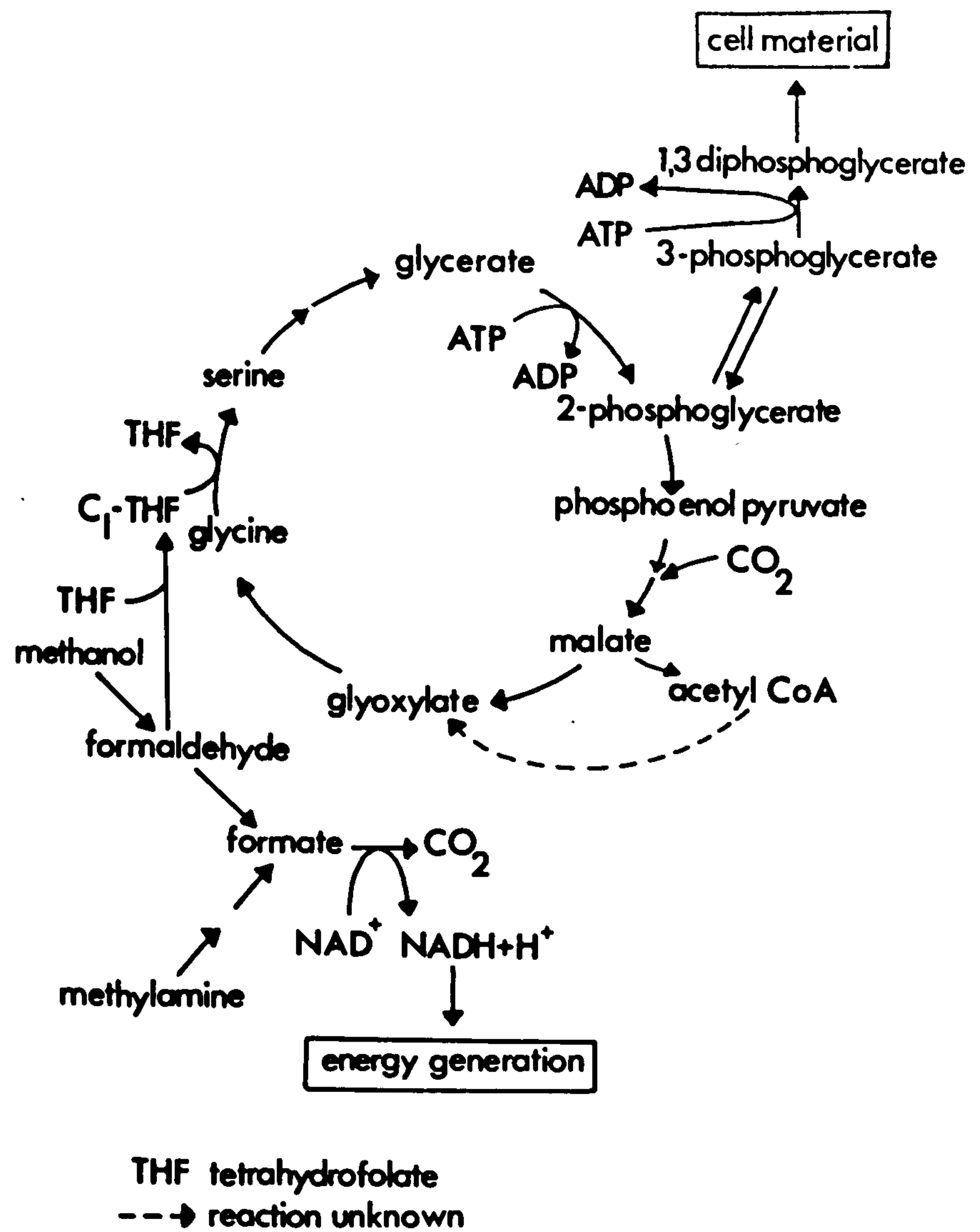


Fig. 3.3 Serine pathway in *Hyphomicrobium* (after Harder and Attwood, 1978).

Hirsch, 1972), (Table 3.1) although ribonucleic acid cistron homology studies (Moore, 1977) do leave the question more open. Furthermore Hyphomonas sp. appear unable to utilise one-carbon compounds (Pongratz , 1957), whereas these are the preferred growth substrate for Hyphomicrobium (Sperl and Hoare, 1971; Attwood and Harder, 1974, 1978). Hyphomicrobium neptunium (Leifson, 1964) isolated from sea water and morphologically resembling Hyphomicrobium vulgare, was proposed as being more closely related to Hyphomonas by DNA-DNA homology sequencing (Hirsch, 1974), and H. indicum (Johnson and Weisrock, 1969) for the same reasoning has also been referred to another genus (Hirsch, 1974) because its morphology is pleomorphic and its G+C ratio is outside the range reported for Hyphomicrobium sp. (Mandel, Hirsch and Conti, 1972).

Tyler and Marshall (1967) isolated a Hyphomicrobium species, designated T37, from hydroelectric pipelines in Tasmania, which showed extensive pleomorphism, depending on the medium employed. Hirsch (1974) proposed that this organism, due to its multistalked forms, should be placed in the genus Pedomicrobium, as described by Aristovskya (1961). Many workers question the validity of the genus Pedomicrobium (Bauld, Tyler and Marshall, 1971; Bauld and Tyler, 1971; Harder and Attwood, 1978). Clearly as the genus Hyphomicrobium is defined to date (Hirsch, 1974), it cannot accommodate the pleomorphism of this organism. This problem could be resolved if the genus was defined so that morphological criteria only applied under optimal growth conditions. Pleomorphism, due to environmental conditions, could then be accommodated (Hirsch and Conti, 1964; Tyler and Marshall, 1967; Shah and Bhat, 1968; Johnson and Weisrock, 1969).

The studies presented here confirm the necessity for modifying the definition of the genus Hyphomicrobium, and appear to invalidate the genera Hyphomonas and Pedomicrobium. These studies concentrate on the characterisation of growth and replication during the cell cycle, and on pleomorphism.

Table 3.1 Base composition (moles % G + C) of known *Hyphomicrobium* strains and prosthecate bacteria

Organism		Neutral CsCl buoyant density g/cm ³	G + C (moles %)
<u>Hyphomicrobium strain</u>			
I ²	EA.617	1.725	66.3
	NQ.521	1.7255	66.8
	WH.563	1.7255	66.8
	MEV.533	1.7245	65.8
II ⁵	NB.762	1.725	64.3
	NM.765	1.725	64.3
	ZV.580	1.7225	63.8
	KB.677	1.7225	63.8
	MY.618	1.7218	62.7
III ⁵	CO.545	1.7195	60.7
	CO.559	1.718	59.2
	MC.650	1.720	61.2
	MC.623	1.719	60.2
	NM.757	1.7195	60.7
	G.522	1.719	60.2
	L.530	1.718	59.2
<u>Hyphomicrobium neptunium</u> ⁵		1.7205	61.7
<u>Hyphomicrobium-H1</u> ⁷		1.7191	60.3
<u>Hyphomonas polymorpha</u> ⁵		1.719 - 1.720	60.2 - 61.2
<u>Rhodopseudomonas palustris</u> ^{1, 6}		1.7235 - 1.725	64.8 - 66.3
<u>Rhodomicrobium vannielii</u> ^{1, 6}		1.7205 - 1.7225	61.8 - 63.8
<u>Pedomicrobium-P1</u> ⁷		1.7190	60.18
<u>Ancalomicrobium</u> ¹		1.7227	64
<u>Prosthecomicrobium</u> ³		1.7245 - 1.7280	65.8 - 69.4
<u>Caulobacter</u> ^{1, 2}		1.7197 - 1.7257	61 - 67
<u>Planctomyces</u> ⁸		—	50.5
<u>Mushroom bacterium</u> ^{4, 7}		1.7257	67.1

1. Dow and Whittenbury (personal communication); 2. Poindexter (1964);
3. Staley (1968); 4. Whittenbury and Nicoll (1971); 5. Mandel et al.,
(1972); 6. Mandel et al., (1971); 7. Lawrence and Dow (in
preparation; 8. Staley (1975).

II. Materials and Methods

(1) Source of organisms

The enrichment and isolation of Hyphomicrobium has been previously described (Section 2.II). Several isolates from oligotrophic water bodies were studied, together with additional strains, Hyphomicrobium C, G and X, which were obtained from W. Harder (University of Gröningen, the Netherlands). A new isolate of Hyphomicrobium, designated Hy. 1, was used in all the studies, unless otherwise stated.

(2) Media

The basal medium was a modification of medium used by Attwood and Harder (1972).

Basal medium (HB): K_2HPO_4 , 1.74 g; NaH_2PO_4 , 1.38 g; $(NH_4)_2SO_4$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.025 mg; $FeCl_2 \cdot 4H_2O$, 3.5 mg in 1 litre of distilled water.

To one litre of this medium, 0.5 ml of a trace element solution (Pfennig, 1969) was added.

For routine culturing, the medium was supplemented with methanol (0.5% v/v) as the carbon and energy source. When growing the organism under anaerobic conditions, KNO_3 (0.2% w/v) was added as the terminal electron acceptor. These were added aseptically to the basal medium. The medium was adjusted to pH 7.0 with \bar{N} NaOH before autoclaving. When solidified media was required, 15 g/l Bactoagar (Difco Laboratories) was added to the liquid media before sterilisation.

(3) Determination of optimal growth conditions

Hyphomicrobium was grown under a variety of different growth conditions. The basal media was modified to give varying concentrations of the carbon and nitrogen source. The pH of the medium was adjusted to give a full range of pH values. The effects of temperature, light and aerobic versus anaerobic conditions were also investigated.

(4) Cultivation of the organisms

Hyphomicrobium was usually maintained anaerobically in subcultures. 100 ml of medium was placed in a 250 ml conical flask (Quickfit B 19) fitted with a ground glass neck, stoppered with a No. 37 standard suba seal (William Freeman and Co. Ltd.). Normally a 1% (v/v) inoculum was used. Cultures were gassed with O_2 free N_2 for ten minutes via syringe needles through the suba seal. Flasks were incubated at $30^\circ C$ on a rotary shaker. Stock cultures were maintained in glass vials at $-70^\circ C$.

Aerobic cultures were maintained by incubating 100 ml cultures in cotton wool plugged, foil wrapped, 250 ml conical flasks, which were agitated vigorously on a rotary shaker.

(5) Determination of growth requirements

Culturing of Hyphomicrobium is possible in completely defined media. To determine if there were any growth requirements or growth stimulants, a culture was grown in regular medium supplemented with combinations of vitamins and amino acids (Table 3.2) (Clowes and Hayes, 1968). Growth was compared after incubation for seven successive days, by optical density measurements. Cultures with no supplementation were used as controls. The effect of peptone and yeast extract on growth was also determined.

(6) Assay for (i) nitrite and nitrate and (ii) hydroxylamine

(i) Nitrite and nitrate

This assay was used routinely on cultures of Hyphomicrobium growing in media supplemented with 0.5% (w/v) KNO_3 as terminal electron acceptor, under anaerobic conditions (Nicholas and Nason, 1957). Samples were filtered through 0.22μ millipore filters (Millipore U.K. Ltd.) and then diluted as required. 0.5 ml of sulphanilamide was added and the mixture shaken. 0.5 ml of 0.02% (v/v) N-1-naphthyl ethylene diamine HCl, which is stored in the dark, was then added. This was mixed, and left to stand for 10 minutes to allow the colour to develop. The optical density at 542 nm was used to determine the level of NO_2^- in the medium.

Table 3.2 Amino acids, vitamins and purine/pyrimidine pools used in the determination of auxotrophic requirements^{††}

Pool	1	2	3	4	5	6
7	Adenine [†]	Biotin [‡] (1)	Phenylalanine [*]	Alanine [*]	Arginine [*]	Leucine [*]
8	Hypoxanthine [†]	Folic acid [‡] (50)	Serine [*]	Cysteine [*]	Ornithine [*]	Glycine [*]
9	Cytosine [†]	Pantothenic acid [‡] (50)	Tryptophan [*]	Threonine [*]	Aspartic acid [*]	Isoleucine [*]
10	Guanine [†]	Pyridoxin [‡] (50)	Tyrosine [*]	Na ₂ S ₂ O ₃ [†]	Proline [*]	Histidine [*]
11	Thymine [†]	Thiamin [‡] (1)	p-Amino benzoic acid [‡] (50)	Methionine [*]	Glutamic acid [*]	Lysine [*]
12	Uracil [†]	Riboflavin [‡] (250)	Nicotinic acid [‡] (250)	Choline [‡] (100)	Inositol [‡] (500)	Valine [*]

[†] Make up at 5 mg/ml

^{*} Make up at 10 mg/ml of L. Form (or 20 mg/ml of DL Form).

[‡] Make up at concentrations shown in parenthesis (µg/ml).

^{††} Concentrations used are derived from J. Lederberg (1950) Meth. Med. Res., 3, 5.

13 Cyanocobalamin[‡](50)

14 Peptone (1 mg/ml)

15 Yeast extract (1 mg/ml)

16 Peptone + yeast extract (1 mg/ml)

Add 1 ml of pool to 100 ml of medium.

Pools 1-12 as described in 'Experiments in microbial genetics', ed. R.C. Clowes and W. Hayes, Blackwell Scientific Publications, Oxford and Edinburgh.

A few grains of zinc dust were added. The mixture was again shaken and left for a further 10 minutes for colour development. This was also read at 542 nm to determine the level of NO_3^- in the medium. Control standards for KNO_3 and KNO_2 were freshly prepared.

(ii) Hydroxylamine

The assay of Magee and Burris(1954) was carried out in order to determine whether any hydroxylamine was present in the medium supplemented with nitrate. 2 ml of culture was taken and made acidic (pH 2-3) with hydrochloric acid. 1 ml of 1% (v/v) alcoholic 8-OH quinoline was added to this, together with 1 ml of 2 N Na_2CO_3 . This mixture was well shaken and then left at room temperature for two hours or alternatively at 60°C for ten minutes. This was subsequently read at 680 nm. Hydroxylamine standards were freshly prepared as were the reagents for the assay.

(7) Assay for CH_3OH levels

The methanol concentration in culture medium was routinely assayed using a Pye Unicam series 104 gas chromatograph fitted with a flame ionisation detector. 5 μ litre samples were analysed at 130°C on a Poropak Q column. Standard solutions of CH_3OH were freshly prepared.

(8) Gas chromatography

Oxygen and nitrogen, and their products, were measured by gas chromatography, using a Pye Unicam series 104 gas chromatograph fitted with a katharometer (thermal conductivity) detector. 0.5 ml of the gaseous phase was assayed. Chromatography was through a 3 metre glass column of 80-100 mesh molecular sieve 5A (Phase Separations Ltd.,) at 50°C with high purity helium carrier gas at a flow rate of 30 ml/min and a detector bridge current of 240 mA. The oxygen to nitrogen content of the samples was calculated from the peak areas using the output traces produced by a linear recorder (Smith Industries, Ltd., Servoscribe recorder) with an associated mechanical integrator (Herbert and Holding, 1972).

(9) Carbon source variations

In certain studies, methanol was replaced by the following which were added aseptically:-

Methylamine hydrochloride	100 mM
Dimethylamine hydrochloride	75 mM
Trimethylamine hydrochloride	75 mM
Sodium formate	100 mM
Formaldehyde	75 mM
Carbon dioxide	10% (v/v with dinitrogen)
Sodium bicarbonate	20 mM
Carbon monoxide	10% (v/v with dinitrogen)
Ethanol	50 mM
Ethanolamine hydrochloride	40 mM
Sodium acetate	30 mM
Sodium lactate	30 mM
Methane	5% (v/v with dinitrogen)

When methanol and methylamine HCl were added together, comparing the effects of these compounds on the morphology of Hyphomicrobium, both were used at a final concentration of 100 mM. The cells were harvested in 0.001 M phosphate buffer before transferring into fresh media supplemented with one of the above carbon and energy sources.

(10) Nitrogen source variations

The following were added individually to HB medium lacking $(\text{NH}_4)_2\text{SO}_4$.

Ammonium chloride	100 mM
Potassium nitrate	100 mM
Potassium nitrite	100 mM
Urea	100 mM
Ammonium phosphate (monobasic)	100 mM
Hydroxylamine hydrochloride	100 mM
Formamide	100 mM
Acetamide	100 mM
Dinitrogen	80%-95% (v/v) with oxygen

Cultures were washed free of any fixed nitrogen and grown either aerobically or in 80% (v/v) argon with 20% (v/v) oxygen. With potassium nitrate, cultures were grown both aerobically and anaerobically, the latter under an atmosphere of argon (100% v/v).

With dinitrogen, the culture was grown with 5%-20% (v/v) oxygen present in the atmosphere. The cells were harvested in 0.001 M phosphate buffer before transferring cells into fresh media, supplemented with one of the above nitrogen sources.

(11) Nitrogen fixation

Fixation of atmospheric nitrogen by Hyphomicrobium sp. was determined by the acetylene reduction assay (Postgate, 1972).

(12) Phosphate concentration variations

Basal media was prepared lacking phosphate. Variable amounts of a stock phosphate solution were added to this medium, to determine whether phosphates affect Hyphomicrobium in growth and morphology. Phosphate solution was prepared from a mixture of 0.1 M $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (35.85 g/l) and 0.1 M $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (15.6 g/l) in proportions to give the required pH of 6.9 .

(13) The effects of heavy metals - manganese and iron

Water samples, collected from various sources (see Section 2.II) were inoculated into:-

- (i) HB media supplemented with 0.02 (w/v) $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (Tyler and Marshall, 1967) as a source of manganese.
- (ii) HB media supplemented with 0.02% (w/v) $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (Shah and Bhat, 1968) or metal paper clips (Hirsch, 1968) as a source of iron.

All cultures were incubated aerobically and anaerobically at 30° C in the dark (Section 2.II). Water samples were inoculated into HB media with 0.5% (v/v) methanol as the carbon source as a control (Attwood and Harder, 1972). The remainder of the water samples were

maintained in three 250 ml flasks with cotton wool plugs. To one was added 0.02% (w/v) $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, to the second a metal clip and the third was left as the control flask. These flasks were left undisturbed for 6 months.

Budding bacteria were isolated from HB enrichments by removing some of the surface pellicle which formed after 2-4 weeks. Streaking generally gave pure cultures of stalked budding bacteria. $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (0.02% w/v) was used as a source of manganese for agar plates, FeS (0.02% w/v) was used as a source of iron for solid media. (Bacto-agar, 1.5% (w/v), Difco Laboratories).

Cells from HB cultures and from natural source 'enrichments' were examined by phase contrast and transmission electron microscopy. Where necessary, manganese deposits were dissolved with 5% (v/v) oxalic acid, iron deposits were dissolved with dilute hydrochloric acid (HCl). The presence of manganese can be shown by its reaction with benzidine hydrochloride to give a heavy white precipitate (Aristovskaya, 1961). Iron oxides can be demonstrated by the resultant blue colour with potassium ferricyanide in HCl (Tyler and Marshall, 1967a). Pure isolates of bacteria were inoculated into HB media supplemented with varying concentrations of (i) $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ and (ii) FeS , to determine quantitatively the effect of these heavy metals on the growth and morphology of stalked budding bacteria.

(14) The effect of inorganic cations and organic amines on morphology

A selection of inorganic cations and organic amines were added to liquid cultures of Hyphomicrobium at various stages in the growth cycle, growing on methanol (125 mM) or methylamine (100 mM), in order to determine whether they would have any effect on pleomorphism of this organism. These compounds included K^+ , Na^+ , NH_4^+ , Fe^{2+} , Mn^{2+} , methylamine.HCl, dimethylamine.HCl, trimethylamine.HCl and ethanolamine.HCl at various concentrations (Ishiguro and Wolfe, 1974).

(15) Growth measurements

Cell growth was measured by:-

- (a) Spectrophotometry, using a Pye Unicam series III SP 500 spectrophotometer at 540 nm.
- (b) Cellular protein determination, by the procedure of Lowry et al., 1951.
- (c) Cell counts, total and viable, using plates and a counting chamber.
- (d) Coulter counting, using a Model ZB1 Coulter counter (Coulter Electronics Ltd., Dunstable, Beds.,) together with a Coulter Channelyzer (C 1000) and XY Recorder II.

(a) Spectrophotometric measurements

Liquid cultures were prepared by filling 3 ml round topped glass cuvettes with inoculated media, stoppering with a small suba seal (No. 17) and gassing briefly with N_2 . These were incubated at $30^{\circ}C$. Samples could be removed periodically with a syringe for microscopic examination, in addition to monitoring the turbidity of the culture at 540 nm.

(b) Cellular protein determinations

1 ml samples were spun down on a microcentrifuge (Quickfit), resuspended in 1 M NaOH and kept at $100^{\circ}C$ for 10 minutes. 0.2 ml was then used for protein estimation against bovine serum albumin as a standard.

(c) Cell counts

(i) Plate counts

0.1 ml samples from cultures were serially diluted 10-fold in HB medium and spread in duplicate on HB plates supplemented with 0.5% (v/v) CH_3OH . Aerobic plates were incubated at $30^{\circ}C$. Anaerobic plates, the medium containing 0.5% (w/v) KNO_3 as electron acceptor, were placed on plastic trays which were placed in nylon bags of 0.05 mm thickness (Portex, Ltd.) along with a 100 ml beaker containing 20 ml saturated pyrogallol (Westmacott and Primrose, 1976). The bags were sealed using a 'Calor Easiseal' (Transatlantic Plastics, Ltd.) and gassed with O_2 free N_2 for 30 minutes via syringe needles.

20 ml of alkali (10% \bar{N} NaOH and 15% (w/v) K_2CO_3) were injected through the bag, the small holes being sealed with Sellotape. A second gassing was given to flush out O_2 originally trapped inside the Petri dishes. To remove the plates, the bag was slit open and the tray removed.

(ii) Counting chamber

Total cell counts were made of these culture dilutions using a Thoma 0.02 mm deep bacterial counting chamber (Gallenkamp, Ltd.).

(d) Coulter counting

Particle counts and the frequency distribution of particle volumes from liquid cultures were determined using a Model ZBI Coulter Counter together with a Coulter Channelyzer C 1000 and XY Recorder II (Smither, 1975). Culture samples were diluted into known volumes of Isoton (Coulter Electronics. Ltd.) which had been previously filtered through a 0.22μ millipore filter (Millipore, Ltd.). Total particle counts were made using a 20μ orifice. Frequency distributions of particle volumes were accumulated in the Channelyzer (Patinkin, 1975). Particle counts were converted to counts/ml and volumes to μm^3 using the formula:-

$$V (\mu m^3) = \left[(\text{Channel No.} \times \frac{\text{window width}}{100}) + \text{B.C.T.} \right] \times T_f$$

where B.C.T. = base channel threshold

T_f = threshold factor.

Latex particles of $0.807 \mu m$ and $1.15 \mu m$ diameter were used as standards (Kubitschek, 1969).

Settings used were as follows:-

B.C.T. = variable

W.W. = 100

Aperture = 20μ

Matching switch = 40 K

Gain = 10

Count range = 10 K

Accumulation time = 1 min

Amplification = variable

Aperture current = variable.

(16) Cell synchronisation

Heterogeneous populations of Hyphomicrobium cells were synchronised by a modification of the sucrose gradient sedimentation method of Mitchison and Vincent (1965). 100 ml of mid-exponential culture was spun down and resuspended in 4 ml of fresh medium. This concentrate was then layered on a 40 ml linear sucrose gradient (5% 10% (w/v) sucrose in HB medium, to minimise physiological shock). This was then centrifuged for 15 minutes at 3,300 rev/min ($\sim 4,200$ g) using the swing out rotor of a MSE Multex centrifuge. A maximum of a third of the gradient could be collected. This contained predominately motile swarmer cells. However, to obtain a maximum of immature swarmer cells, 0.1 ml was removed from the uppermost fractions of the gradient, which gave 1×10^7 cells/ml. This was sufficient for slide and liquid culture studies but restricted biochemical studies due to the lack of cell biomass. The degree of synchrony was assessed by light microscopy and Coulter Counter studies, using as controls the heterogeneous cell population, and other fractions further down the sucrose gradient towards the pellet at the bottom.

(17) Slide culture

Slide cultures were prepared, both aerobically and anaerobically, according to the study being undertaken.

(a) Anaerobic slide cultures

Slides and coverslips were cleaned by soaking in chromic acid overnight, washed in distilled water and finally sterilised by immersing in ethanol and flaming. The appropriate nutrient agar (HB) was boiled in a water bath for 30 minutes, with nitrogen bubbling into the molten agar. Two drops of agar were pipetted on to a microscope slide positioned inside a perspex box, which was continually under an atmosphere of O_2 free N_2 . The agar was covered with a coverslip in order to give a thin, even layer of agar on the slide. After five minutes the coverslip was carefully removed from the agar, and the agar was inoculated with a drop of culture. A fresh coverslip was applied, the slide culture was removed from the anaerobic box, and rapidly sealed with a Paraffin/Vaseline mixture.

(b) Aerobic slide cultures

The preparation was again carried out aseptically ; however, there was no need for a special environment, and so this was done in a sterile glass Petri dish. The same procedure was carried out, but this time a trough was cut in the agar to ensure a sufficient supply of oxygen. A small drop of culture was applied to each agar half, to avoid flooding the trough, and the system sealed as before.

(18) Spheroplast formation

Spheroplasts of Hyphomicrobium were produced by a modification of the method described by Schmidt and Stanier (1966). The sodium salt of benzyl penicillin (Crystopan, Glaxo, Ltd.) was dissolved in HB medium to give a final concentration of 10,000 units/ml. 0.2 μ g/ml of lysozyme was also added. The medium was supplemented with 8% (w/v) polyethylene glycol as an osmotic stabiliser. Incubation was at 30⁰ C, for liquid and slide culture. Synchronous and heterogeneous populations of Hyphomicrobium were removed at 1 hour intervals, and subjected to penicillin treatment for (a) 1 hour and (b) 2 hours. The penicillin treatment was terminated by the addition of 1 ml of penicillinase (Koch-Light Laboratories Ltd.) at 35⁰ C, pH 7.0 (Park et al., 1957). Controls were carried out by growing cells under the same conditions, with and without penicillin for the full eight hours.

(19) Light microscopy

All cultures were examined by phase contrast microscopy as described in Section 2.II. For slide culture studies, the Olympus microscope was maintained at 30⁰ C or the Leitz microscope was used, for which there was a controlled heating stage.

(20) Transmission electron microscopy

Washed samples of cells were dried on to Formvar coated copper grids, having previously been fixed in OsO₄ vapour for 5 minutes. The preparations were either negatively stained or metal shadowed, as previously described (Section 2. II).

(21) Sectioning

The fixation and embedding procedures of Ryter and Kellenberger (1958) and Spurr(1969) were followed for thin sections as described in Section 2. II.

(22) Bacteriophage for Hyphomicrobium

Presumptive 'phage preparations from freshwater and sewerage concentrated by means of adsorption filtration (N. Seeley, personal communication) were tested against Hyphomicrobium isolates. To determine the lytic ability, serial dilutions of the presumptive 'phage preparations were mixed with Hyphomicrobium isolates in HB medium top agar (0.75% w/v) which was then poured over a layer of HB medium bottom agar (1.5% w/v) in the standard overlay technique. After 3, 6 and 9 days the lawns were checked for phage plaques.

Alternatively freshwater and sewerage samples were made into a complete growth medium and were inoculated directly with eight different isolates of Hyphomicrobium, and incubated at 30° C with shaking. After incubation, the cultures were centrifuged and filtered through Millipore filters (0.45 µm and 0.22 µm), concentrated down and serial dilutions were used for plaque assays as described above.

(23) Continuous culture studies

The growth vessel was a Quickfit FV1L culture vessel and flanged lid, with a working volume of 500 ml (Fig. 3.3). Culture temperature was controlled by circulating water around a water jacket (a large glass tank) using a Churchill thermocirculator. The culture was agitated by means of a magnetic follower and stirrer. pH control was facilitated by means of an electrode coupled to an industrial meter and controller. Sterile filtered air or oxygen free nitrogen was bubbled through the culture. The fermenter vessel, with all accessories was sterilised at 121° C for 30 minutes. Fresh medium refills were pumped into the reservoir from a previously sterilised 20 litre supply via 0.3 µm 'Whatman Gamma' in line filters. Any supplements were added to the 20 litre media pot via reservoirs. Samples were taken from the overflow using a sampling hood and sterile Universal bottles.

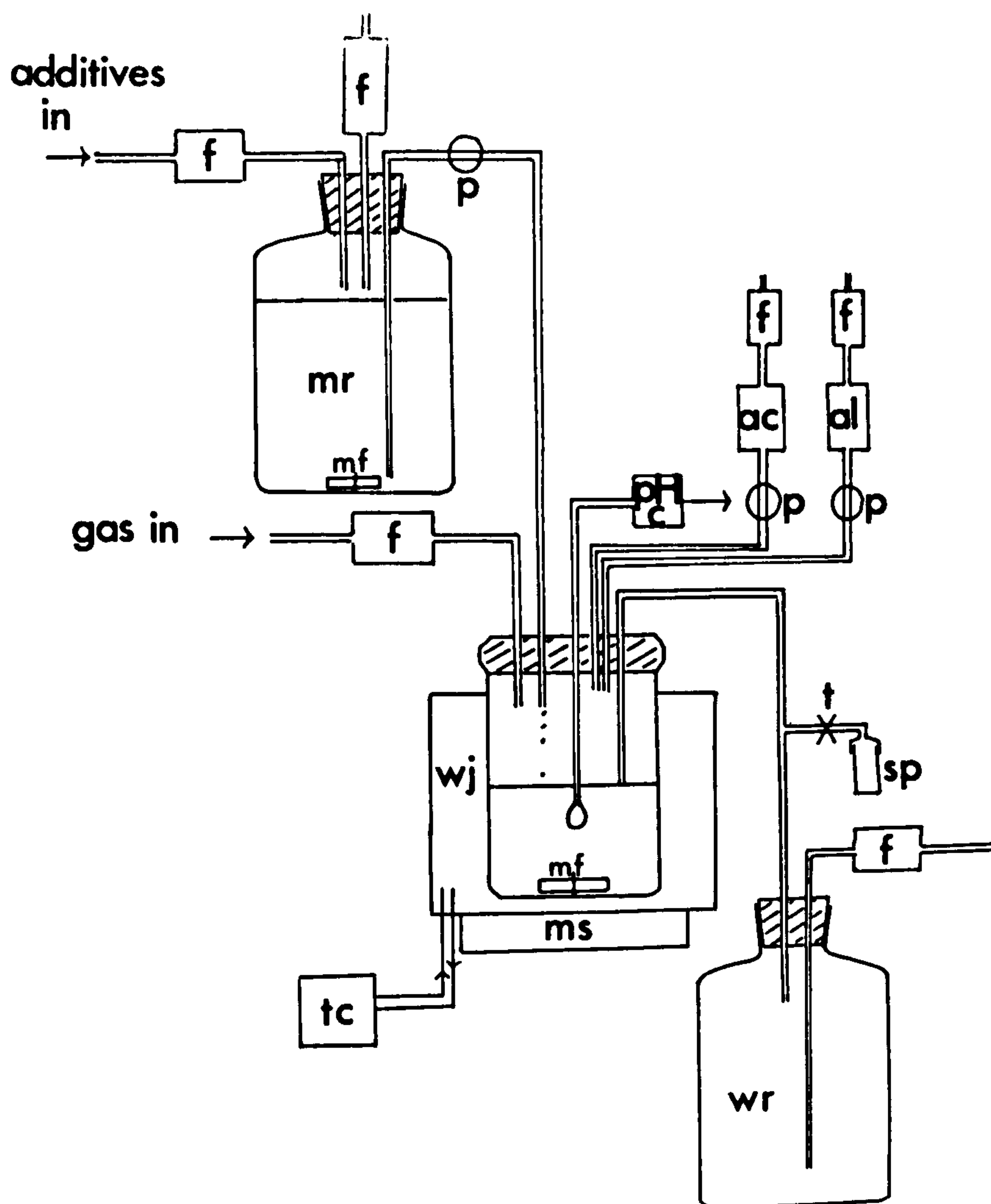


Fig. 3.4. Continuous culture apparatus

wj = water jacket

ms = magnetic stirrer

mf = magnetic flea

tc = temperature control

f = filter

ac = acid

al = alkali

pHc = pH control

p = peristaltic pump

sp = sampling port

mr = medium reservoir

wr = waste reservoir

(24) Uptake and incorporation of label

Determination of the incorporation of radioactive CH_3OH and $\text{CH}_3\text{NH}_2 \cdot \text{HCl}$ was carried out by incubating cells in the presence of such material labelled with ^{14}C . Label was used at a final concentration of $100 \mu\text{Ci/ml}$. $^{14}\text{CH}_3\text{OH}$ had a specific activity of 55.5 mCi/mmol , and $^{14}\text{CH}_3\text{NH}_2 \cdot \text{HCl}$ of 60.0 mCi/mmol . Media was prepared in the usual manner, using $0.1\% \text{ v/v}$ carbon source, as cold substrate where necessary. Growth of the cells was monitored spectrophotometrically. Incorporation of the label was determined by periodically removing samples of 0.4 ml of culture, in duplicate, into 10 ml of $5\% \text{ (w/v)}$ trichloroacetic acid (TCA), kept at 4°C , for 30 minutes. Samples were filtered on 2.5 cm Whatman GF/C glass fibre filters, washed twice with cold $10\% \text{ (w/v)}$ TCA, then distilled water and finally ethanol, and dried for 2 hours. These were subsequently counted using a Packard Tri-Carb scintillation counter.

(25) DNA preparation, determination of base composition

DNA samples were prepared as described in Section 2. II for analytical ultracentrifugation, using caesium chloride gradients.

(26) DNA-DNA homology studies - duplex formation

DNA samples were obtained by phenol extraction (Grossman, 1968). ^{32}P labelled DNA was prepared by growing cultures in phosphate free HB medium, containing $30 \mu\text{Ci/ml}$ ^{32}P -phosphate (specific activity 80 Ci/mg phosphorous). The various DNA species were heat denatured by boiling in 0.12 molar phosphate buffer for 8 minutes. The reaction mixture consisted of $30 \mu\text{l}$ cold DNA, $20 \mu\text{l}$ ^{32}P -DNA, $1.25 \mu\text{l}$ 4.8 molar phosphate buffer, $48.75 \mu\text{l}$ water, and was reacted in 0.12 M phosphate buffer at 69°C and then passed down a hydroxyapatite column maintained at 65°C . Single stranded DNA was eluted in 0.12 M phosphate buffer, whilst double stranded DNA was eluted in 0.3 M phosphate buffer. The eluted samples were counted using a Pakard Tri-Carb scintillation counter.

(27) Enzyme assay - hydroxypyruvate reductase

(a) Extract preparation

Hydroxypyruvate reductase was assayed for in Hyphomicrobium, the presumptive Pedomicrobium and an isolate of mushroom shaped bacterium, the last as a control study. Cultures were grown on methanol, methylamine or formate, all at concentrations of 0.2% (w/v). Mushroom shaped bacterium would utilise these one-carbon compounds, but at a lower rate compared to Hyphomicrobium. Cells were harvested in log phase of growth by centrifugation at 4°C, washed twice and resuspended in 4 ml of 0.05 M phosphate buffer (pH 7.0) containing 2 mM 2-mercaptoethanol (Attwood and Harder, 1973). Cell suspensions were disrupted by sonication for 3 x 1 minute at 0°C, using an ultrasonic disintegrator (M.S.E. Model 150W) using a power output of 60 W at 25 KHz. Sonicates were then spun for 2 minutes, and the clear supernatant retained (the soluble protein fraction). These fractions were frozen in dry ice/alcohol mixture and stored at -20°C until required.

The protein concentration of the soluble protein fractions (SPF) was determined by the method of Lowry et al. (1951). Bovine serum albumin was used as the standard.

(b) Hydroxypyruvate reductase assay

This enzyme was assayed according to the method of (i) Blackmore and Quayle (1970) and (ii) Large and Quayle (1963).

(i) Silica cuvettes (3 ml, light path 1 cm) contained 100 μ mol of sodium acetate buffer (pH 4.5), 0.4 μ mol of NADH and 2 μ mole of lithium hydroxypyruvate in a total volume of 3 ml. Extract was added and the decrease in extinction at 340 nm was measured in a recording spectrophotometer against a blank containing all the components except NADH. The rate was corrected for the oxidation of NADH by the extract in the absence of substrate. One unit is defined as that amount of enzyme which catalyses the oxidation of 1 μ mol of NADH in 1 minute under the assay conditions.

(ii) Silica cuvettes contained 100 μ mol of phosphate buffer (pH 7.5 or 6.5), 0.4 μ mol of NADH and extract in a total volume of 3 ml. Lithium hydroxypyruvate (2 μ mol) was added and the decrease in extinction at 340 nm was measured against a blank containing buffer and extract. The rate was corrected for oxidation of NADH by extract in the absence of substrate.

(28) Polyacrylamide gel electrophoresis

(a) Soluble protein fractions. - These were prepared as described for the enzyme assay.

(b) Gradient slab gels (exponential), 10%-30% (w/v) acrylamide

Sample preparation :- Samples (SPF) were prepared as described above.

Gel reagents:-

High bis-Acrylamide stock:-	Acrylamide	60 g	
	bis-Acrylamide	1.6 g	100 ml
	water		
Low bis-Acrylamide stock:-	Acrylamide	60 g	
	bis-Acrylamide	0.3 g	100 ml
	water		
Stacking gel acrylamide:-	Acrylamide	10 g	
	bis-Acrylamide	0.5 g	100 ml
	water		
Lower gel buffer(pH 8.8):-	Tris	36.6 g	
	HCl (conc.)	4.13 ml	100 ml
	water		
Stacking gel buffer(pH 6.8):-	Tris	5.98 g	
	HCl (conc.)	4.13 ml	100 ml
	water		
Running buffer:-	Stock	200 ml	
	10% (w/v) sodium lauryl sulphate	10 ml	
	water	790 ml	

(where stock buffer = 30.2 g/L Tris + 144 g/L glycine).

Initiator:- freshly prepared 10% (w/v) ammonium persulphate.

T.E.M.E.D.:- NNN'N' tetramethylethylenediamine.

Gel mixtures:-

10% (w/v) gel mix	High bis-Acrylamide stock	8.3 ml
	Water	34.9 ml
	Lower gel buffer	6.25 ml
	10% Sodium laurylsulphate (w/v)	0.5 ml
	T.E.M.E.D.	10 μ l

30% (w/v) gel mix	Low bis-Acrylamide stock	10 ml
	Glycerol, 75% (w/v)	7.3 ml
	Lower gel buffer	2.5 ml
	10% Sodium lauryl sulphate (w/v)	0.2 ml
	T.E.M.E.D.	4 μ l

Both gel mixes were swirled and degassed. Initiator was then added (40 μ l to the 30% (w/v) gel mix and 100 μ l to the 10% (w/v) gel mix).

Stacking gel mix	10% stacking gel acrylamide (w/v)	3.0 ml
	Water	4.4 ml
	Stacking gel buffer	2.4 ml
	10% Sodium lauryl sulphate (w/v)	0.1 ml
	T.E.M.E.D.	5 μ l
	10% (w/v) ammonium persulphate	0.1 ml

(c) Gel preparation and electrophoresis .- Slab gels (200 x 235 x 1.5 mm) were made by using the discontinuous system described by Laemmli (1970), using a Tris-glycine (pH 8.4) electrophoresis buffer, a polyacrylamide stacking gel (pH 6.8) and a resolving gel containing Tris HCl at pH 8.8

Gradient gels were made using 20 ml of high concentration mixture (30% (w/v) gel mix) and 50 ml of low concentration mixture (10% (w/v) gel mix) in a constant volume mixing chamber, into which was pumped the 50 ml of 10% (w/v) gel mix. The gradient was thus exponential, running from 10% (w/v) at the top to 30% (w/v) at the bottom. This gradient was then overlaid with stacking gel mix, and sample wells were made using a slot former with Teflon teeth. 6% (w/v) sucrose, 0.1 ml of 10% (w/v) sodium lauryl sulphate, 10 μ l 8-mercaptoethanol, and 20 μ l of 0.1% (w/v) bromophenol blue tracker dye were added per 0.5 ml of soluble protein fraction, and the mixture was heated to 100^o C for 3 minutes. Samples were loaded on to the gel; electrophoresis was at 19 mA constant current until the blue marker had reached the base of the gel (12-14 hours), using a Shandon Southern SAE 2761 power pack.

(d) Gel staining:- Gels were completely submerged in 0.1% (w/v) coomassie blue, 45% (v/v) methanol and 10% (v/v) acetic acid for a minimum of 3 hours, and destained in 45% (v/v) methanol, 10% (v/v) acetic acid for 3-4 hours, 20% (v/v) isopropanol, 10% (v/v) acetic acid for 6 hours and finally in 10% (v/v) isopropanol, 10% (v/v) acetic acid to completion.

Gel pattern recording:- Gels were photographed from above by sandwiching between glass plates, which were then placed on a glass illuminator (Industrex X-ray illuminator, Model 2, Kodak Ltd., London) using a Pentax SP 500 camera with Kodak Panatomic X film (ASA 32).

(e) Standards:- Standards when used included bovine serum albumin, m.w. 67,000; ovalbumin, m.w. 43,000; γ -globulins, m.w. 25,000 and 50,000; myoglobin, m.w. 16,890; cytochrome C, m.w. 13,400.

Relative fronts (r.f.) were measured relative to the bromophenol blue front.

3.III Results and Discussion

1. Growth and Physiology

(a) Occurrence

Hyphomicrobium can be readily isolated from soil and from both oligotrophic and eutrophic waters (Section 2.II). The concentration of carbon to nitrogen levels from the inoculum and medium determined the ease of isolation. A low nitrogen level together with a high carbon inoculum resulted in a very slow development of Hyphomicrobium from sources relatively rich in organic material, i.e. eutrophic waters, however, an increase in the nitrogen concentration eventually brought through this organism. Therefore, although ubiquitous, Hyphomicrobium was more readily isolated from oligotrophic environments.

(b) Growth conditions

When cultures of Hyphomicrobium were grown under standard conditions (Section 3.II) good growth in nine isolates tested (including C, G and X from M. Attwood and W. Harder) was evident after three days incubation (> 1 mg protein/ml medium, Lowry et al., 1951). If the level of carbon and nitrogen in the medium were both lowered (0.05% (w/v) CH_3OH and 0.02% (w/v) nitrate), growth occurred, although cultures took eight days to reach a comparable concentration of protein. When the carbon and nitrogen levels were increased to 2% (v/v and w/v respectively), growth was very slow, cultures taking up to 24 days to reach the growth of control cultures. Any imbalance of carbon/nitrogen caused slow growth and pleomorphism (Section 3.III.4).

When the media was adjusted to below pH 6.0 or above pH 8.0, growth was retarded. The pH optimum was 7.0, in accordance with previous workers (Sperland Hoare, 1971; Attwood and Harder, 1972).

The temperature range for culturing Hyphomicrobium was within the range 25°C - 35°C , with optimum growth at 30°C . Growth also occurred between 5°C - 50°C , although this appeared to vary with individual isolates, however, one isolate did grow optimally at 45°C .

The effect of light on cultures of Hyphomicrobium was fairly pronounced (Hirsch and Conti, 1964a). Normally cultures were grown on a rotary shaker, where the incident light intensity was 100 lux, compared to 600 lux in the open laboratory. Cultures grew normally with an incident light intensity of up to 800 lux, but above this level growth was retarded, and the cells became pleomorphic (Fig. 3.5). No wall effects were observed in static culture, until the light intensity was greater than 1000 lux; this then appeared to suppress uniform growth of the culture. In this case there was some distortion in the morphology, with the cells enlarging and the stalks appearing stunted (Fig. 3.5).

(c) Carbon and nitrogen sources, and metabolism

Of all the carbon sources tested, only methylamine and formate could be substituted for methanol to give good growth of the nine isolates tested (Figs. 3.6, 3.7), although formate consistently gave poorer growth yields (cf. Harder and Attwood, 1978). Ethanol, acetate and formaldehyde were utilised very slowly, but growth in these instances may not have been solely due to these substrates, as Hyphomicrobium has been shown to be capable of growing oligocarbophilically (Kingma-Boltjes, 1936; Hirsch and Conti, 1964b). Controls with no added carbon grew poorly under anaerobic conditions, but grew reasonably well under aerobic conditions, presumably utilising gaseous carbon compounds in the laboratory atmosphere, which, over a period of time, could penetrate through the cotton wool plug. Some growth was observed on dimethylamine hydrochloride and trimethylamine hydrochloride, however, the cultures became very acidic. With gaseous carbon compounds, pellicle formation was only observed with methane, which gradually appeared after eight weeks incubation; the cells were very elongated, with stalks greater than 10 μm in length (cf. Namsaraev and Zavarzin, 1972). Poor culture growth was observed on C-2 compounds, disagreeing with studies by Harder, Matin and Attwood (1975) and by Harder and Attwood (1978) who obtained good growth on acetate and ethanol. No growth was obtained on higher carbon compounds (C_3 - C_6), confirming the studies of Harder and Attwood (1978) with the exception that they found that 3-hydroxybutyrate would act as a carbon source for the Hyphomicrobium sp. they tested.

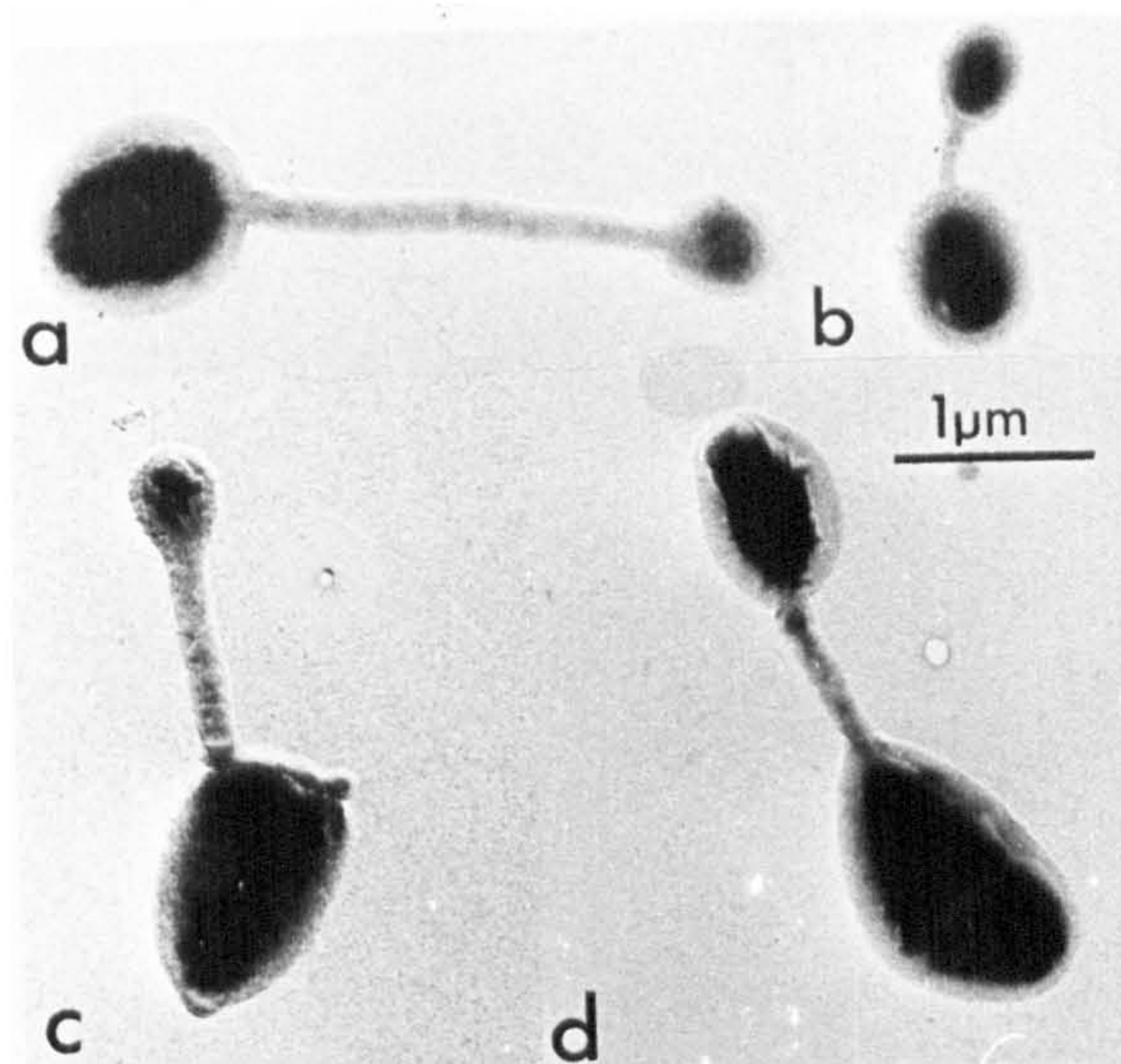


Fig. 3.5 The effect of light on Hyphomicrobium. Cultures grown at 2000 lux. Gold/Palladium shadowed.

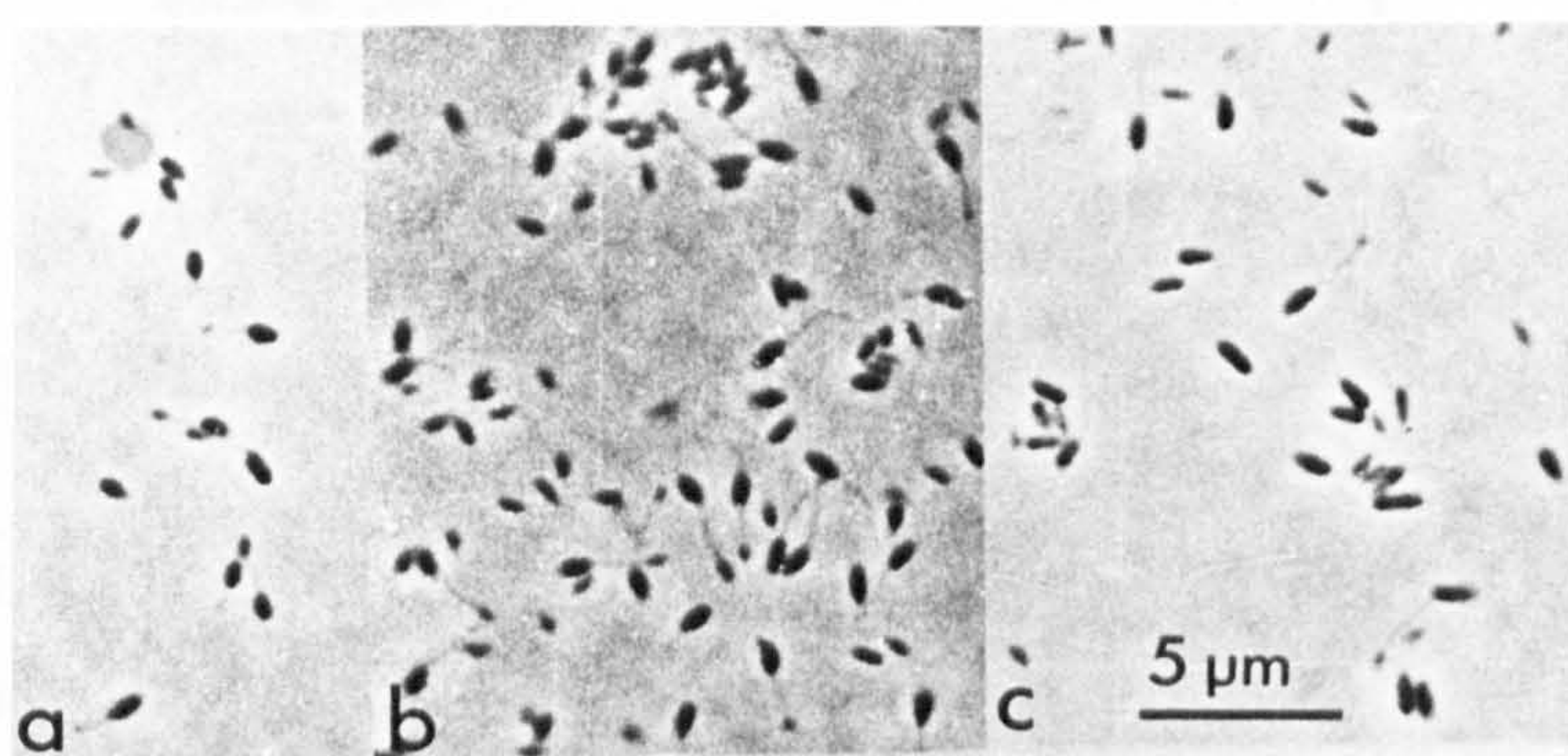


Fig. 3.6 Light micrographs of heterogeneous population of Hyphomicrobium growing on (a) formate, (b) methylamine and (c) methanol in batch culture.

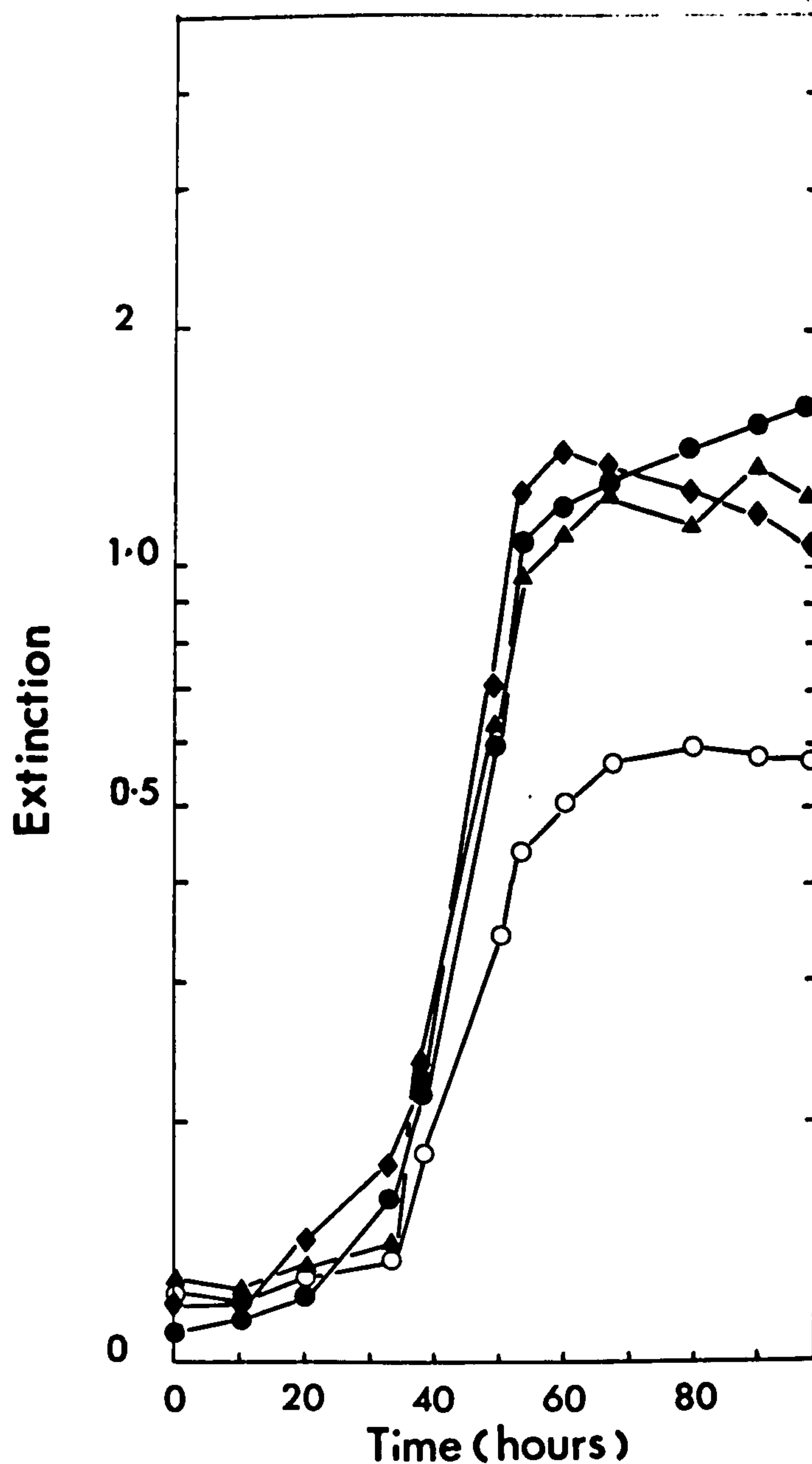


Fig. 3.7 Growth of *Hyphomicrobium* on methanol (—◆—), methylamine (—▲—), methanol and methylamine (—●—) and formate (—○—). Growth was consistently poor on formate. Optimal growth was obtained with methanol or a mixture of methanol and methylamine.

As a nitrogen source, $(\text{NH}_4)_2\text{SO}_4$ was routinely used. Methylamine hydrochloride and nitrate were also readily used as nitrogen sources. Nitrite, at low levels ($< 0.05\%$ w/v) was utilised by Hyphomicrobium, but high levels of nitrite and also nitrate often led to pleomorphic forms developing (Section 3.III.4).

No significant levels of hydroxylamine were detected in culture medium supplemented with high levels of nitrate as it underwent denitrification. Urea could also support growth of this bacterium. Formamide and acetamide were also used as nitrogen sources, but they both caused variations in the appearance of the cells (Fig. 3.9). Fixation of nitrogen was not substantiated by the acetylene reduction test (Postgate, 1972), any turbidity in the medium was attributed to traces of fixed nitrogen compounds in components of the culture medium.

(d) Phosphate effects

In media, with no added phosphates, growth was poor (0.1 mg protein per ml or less), and the cells were elongated, with stalks up to $20\ \mu\text{m}$ in length (Fig. 3.8). In the routine medium, phosphate is in excess ($> 100\ \text{mM}$) and so it is assumed that at no time is phosphate limiting in the culture medium. If discreet amounts of phosphate are added to phosphate free medium, the morphology of the cells alters with the cells becoming ovoid rather than elongated and the stalk length reducing significantly.

(e) Growth requirements

As far as could be determined, none of the combinations of amino acids and vitamins supplemented into the basal medium stimulated growth. Further, the presence of peptone and/or yeast extract in the medium appeared to retard the development of the culture.

Hirsch and Conti (1964b) have shown that there is a need for manganese, molybdenum, calcium, iron and phosphate in the medium, so trace elements were routinely employed in the basal medium.

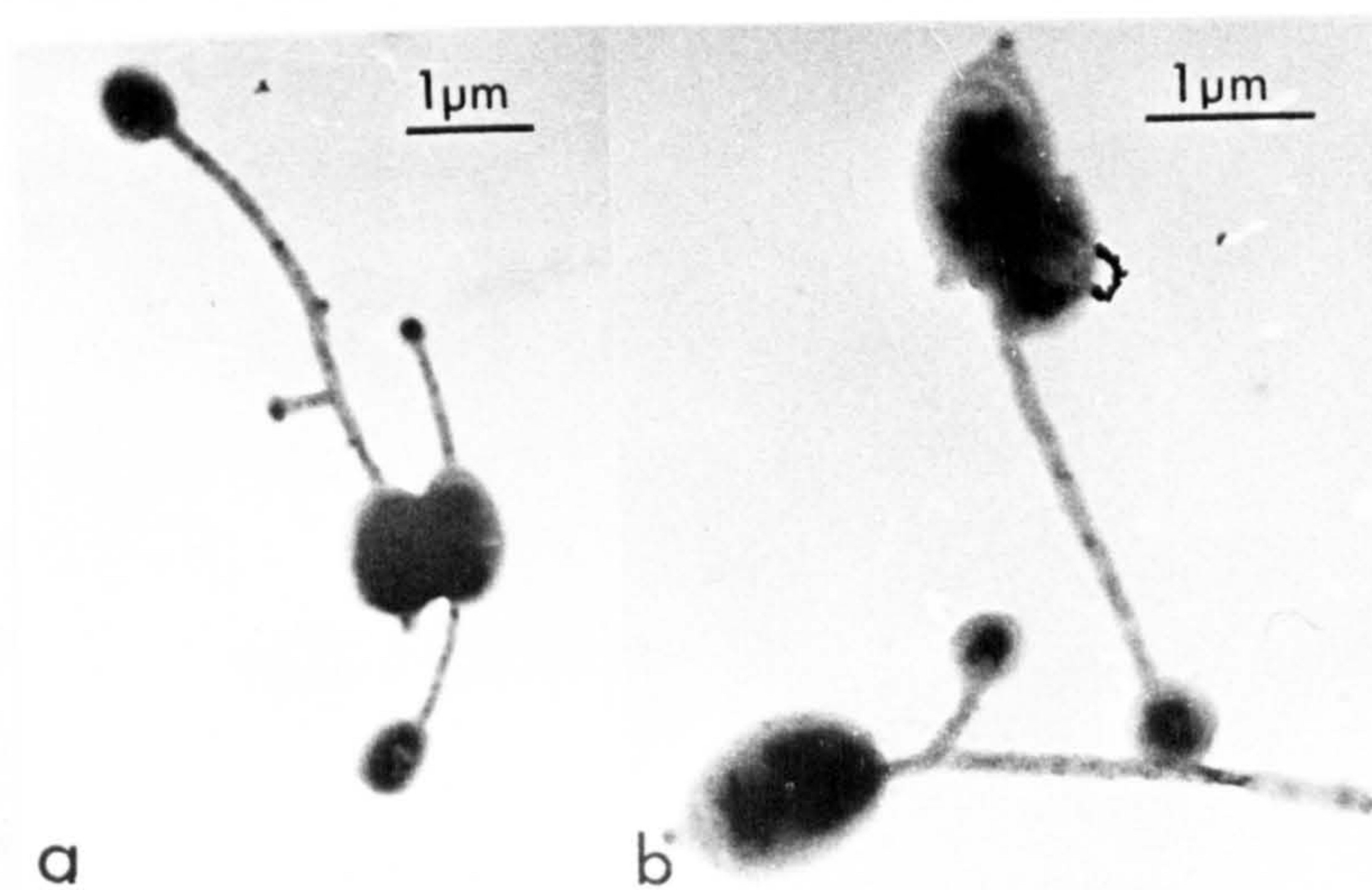


Fig. 3.8 Hyphomicrobium , grown in phosphate-free medium. Stalks became very elongated and branched.

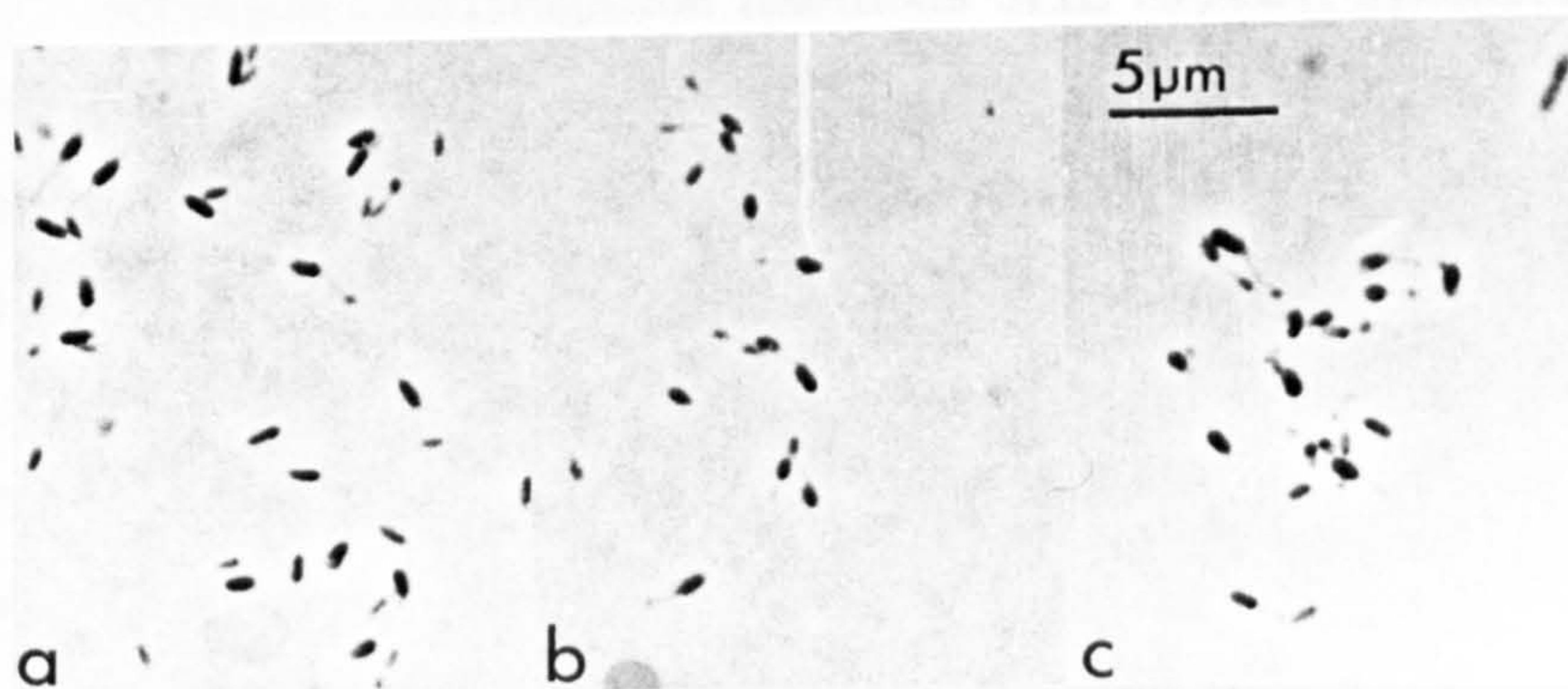


Fig. 3.9 Growth of Hyphomicrobium on various nitrogen sources (a) nitrite, (b) formamide and (c) acetamide.

(f) Aerobic and anaerobic growth

The addition of nitrate as a terminal electron acceptor into the basal medium enabled Hyphomicrobium to be grown anaerobically (Sperl and Hoare, 1971; Attwood and Harder, 1972; Uebayasi and Tonomina, 1976). As the nitrate undergoes denitrification, with considerable gas production (Uebayasi and Tonomina, 1976), the medium was assayed to determine quantitatively, the amount of nitrate remaining, and also the presence of any nitrite groups, as nitrate is denitrified via nitrite and nitrous oxide to dinitrogen. Nitrous oxide levels were monitored by gas chromatography (Section 3.II.8). When nitrate levels exceeded 0.5% (w/v) in the culture medium, nitrous oxide could be detected up to one part in a hundred, the gas phase being dinitrogen.

Cultures of Hyphomicrobium grown anaerobically gave comparable growth to aerobic cultures (≥ 1 mg protein/ml in three days using a 1% (v/v) cell inoculum). Anaerobic cultures proved very convenient for slide culture studies and cuvette culture monitoring, minimising any contamination problems. However, nitrate levels above 0.5% (w/v) (Section 3.III.4) affected the cellular morphology.

2. Life cycle and morphology

(a) Synchronisation

Using differential centrifugation (Methods 3.II.16) cell synchronisation greater than 95% was confirmed by both light microscopy and Coulter counter analysis (Figs. 3.10, 3.11, 3.12). Growth of the synchronised cell population was followed by monitoring the extinction at 540 nm, cell counts and cell volume measurements, and by light microscopy using slide cultures. Extinction monitoring showed that the cells initiated growth as soon as they were inoculated into fresh medium (Fig. 3.13), however slide cultures exhibited about a one hour lag before the cells started to develop. This lag may have been due to the transition from liquid to solid media, rather than due to any physiological disruption due to the synchronisation procedure. The swarmer cells matured

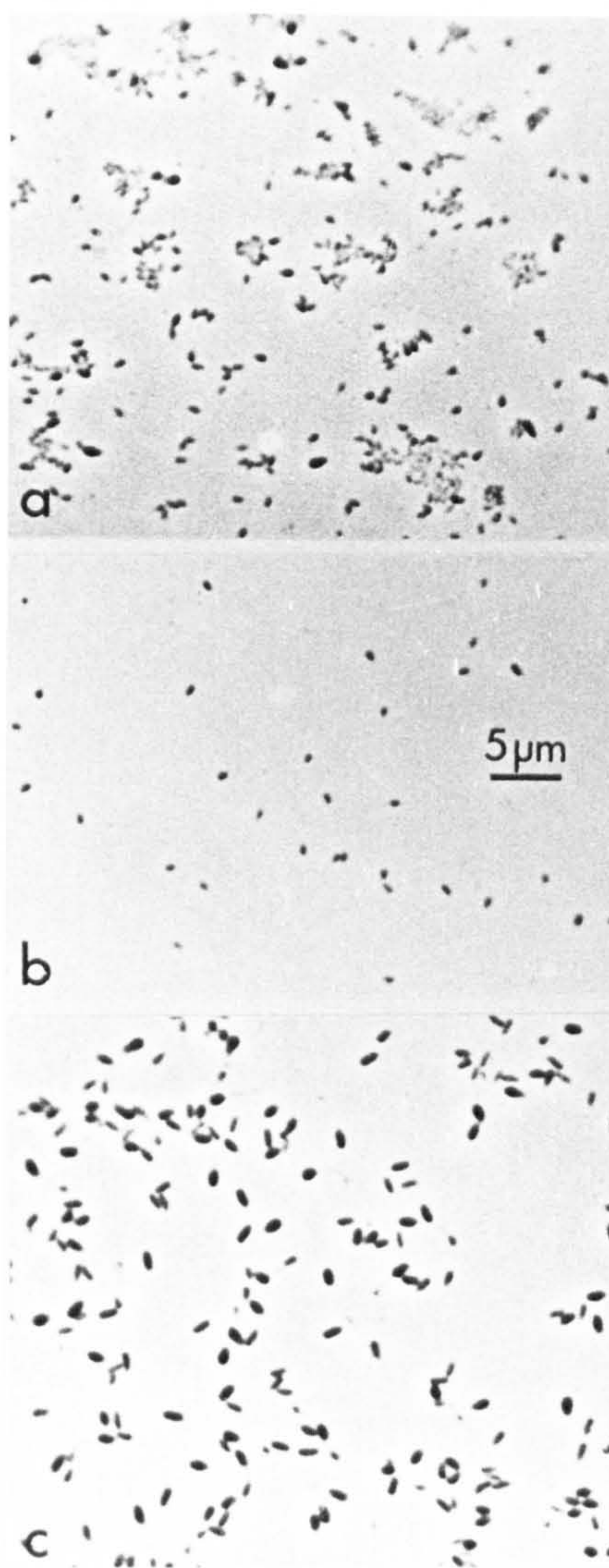


Fig. 3.10 Light micrographs of synchronised Hyphomicrobium.

- (a) Swarmer cells from the first third of the gradient (note heterogeneity in size).
- (b) Immature swarmer cells taken from the top of the gradient.
- (c) Heterogeneous population of Hyphomicrobium.

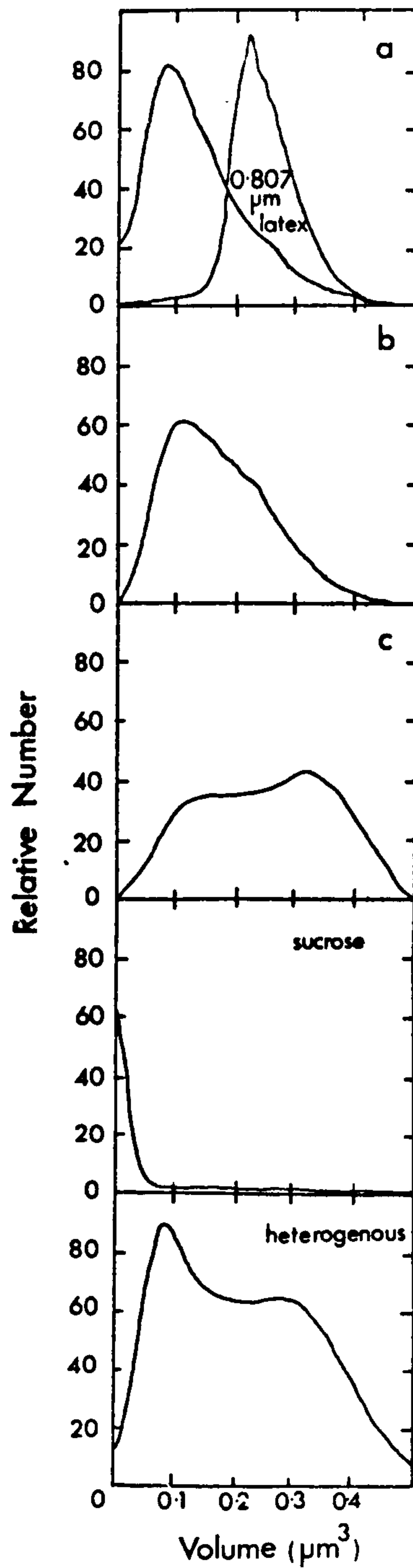


Fig. 3.11 Coulter counter traces taken from fractions down the sucrose gradient. (a) top of gradient, (b) first third of gradient, (c) bottom third of gradient, (d) sucrose, (e) heterogeneous population. 0.807 μm latex particle as standard. $1/\text{Aperture current} = \frac{1}{2}$, $1/\text{amplification} = \frac{1}{2}$, base channel threshold = 5).

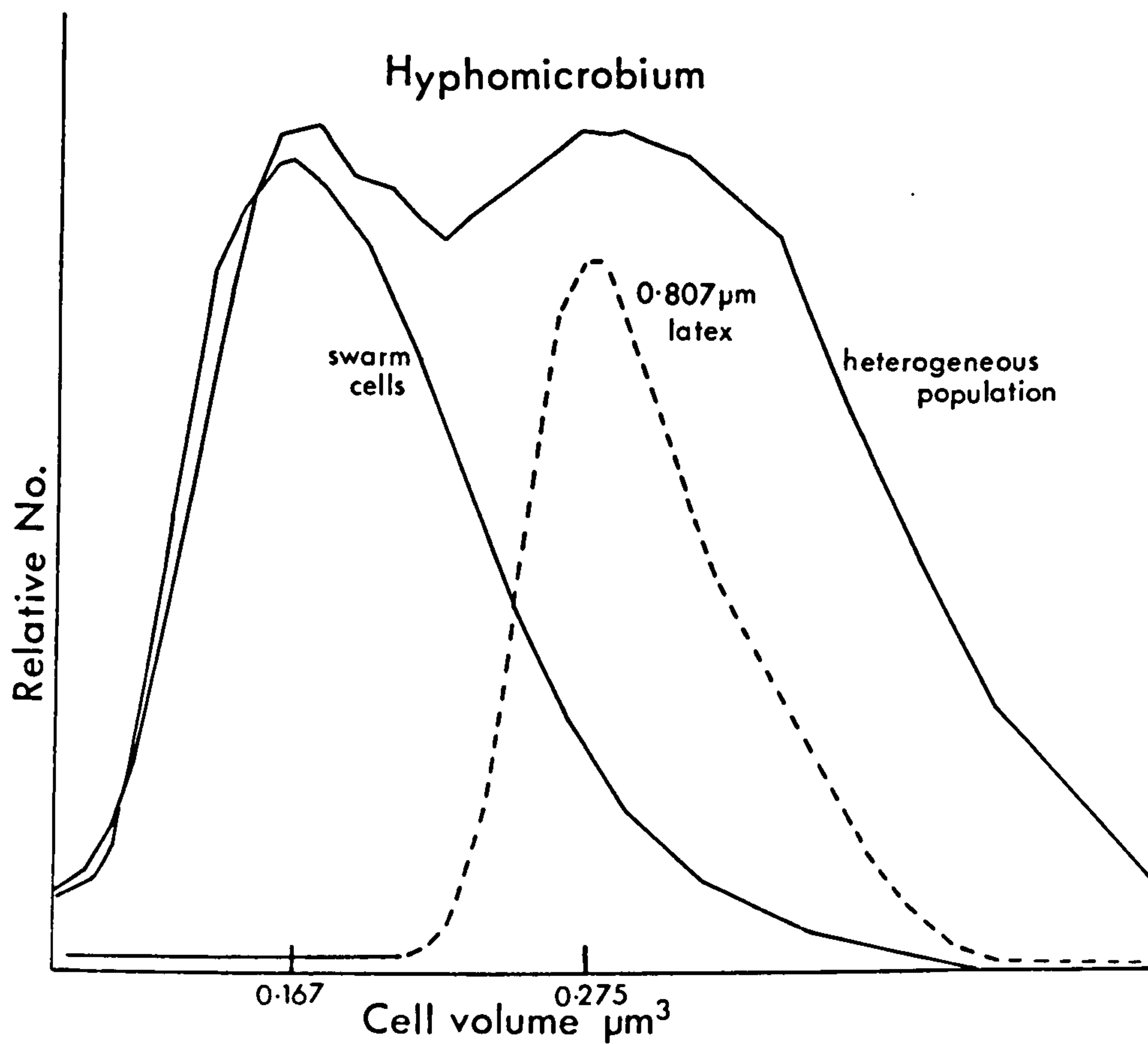


Fig. 3.12 Coulter traces of immature swarmer cells and the heterogeneous population. $1/\text{Aperture current} = 1, 1/\text{amplification} = 1/8$, base channel threshold = 5). 0.807 latex particle as standard.

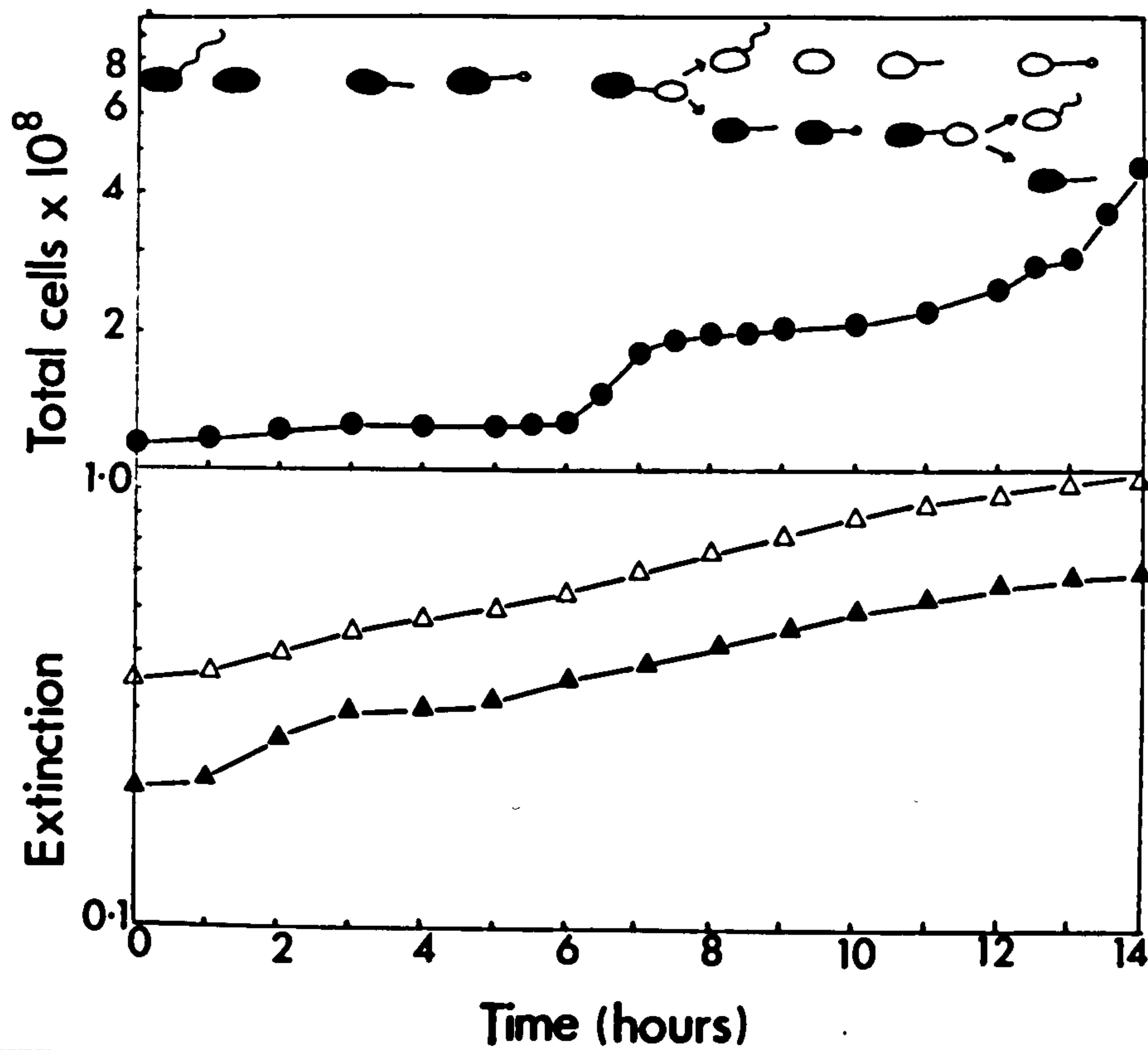


Fig. 3.13 Development of the synchronised population. Top graph relates to cell numbers, determined by Coulter counter, with light microscope observations. Extinction measurements of synchronised and heterogeneous populations, in lower graph, illustrated that the stalked cells correspond to the levelling off between 3 and 5 hours.

heterogeneous —△—
 synchronised —▲—

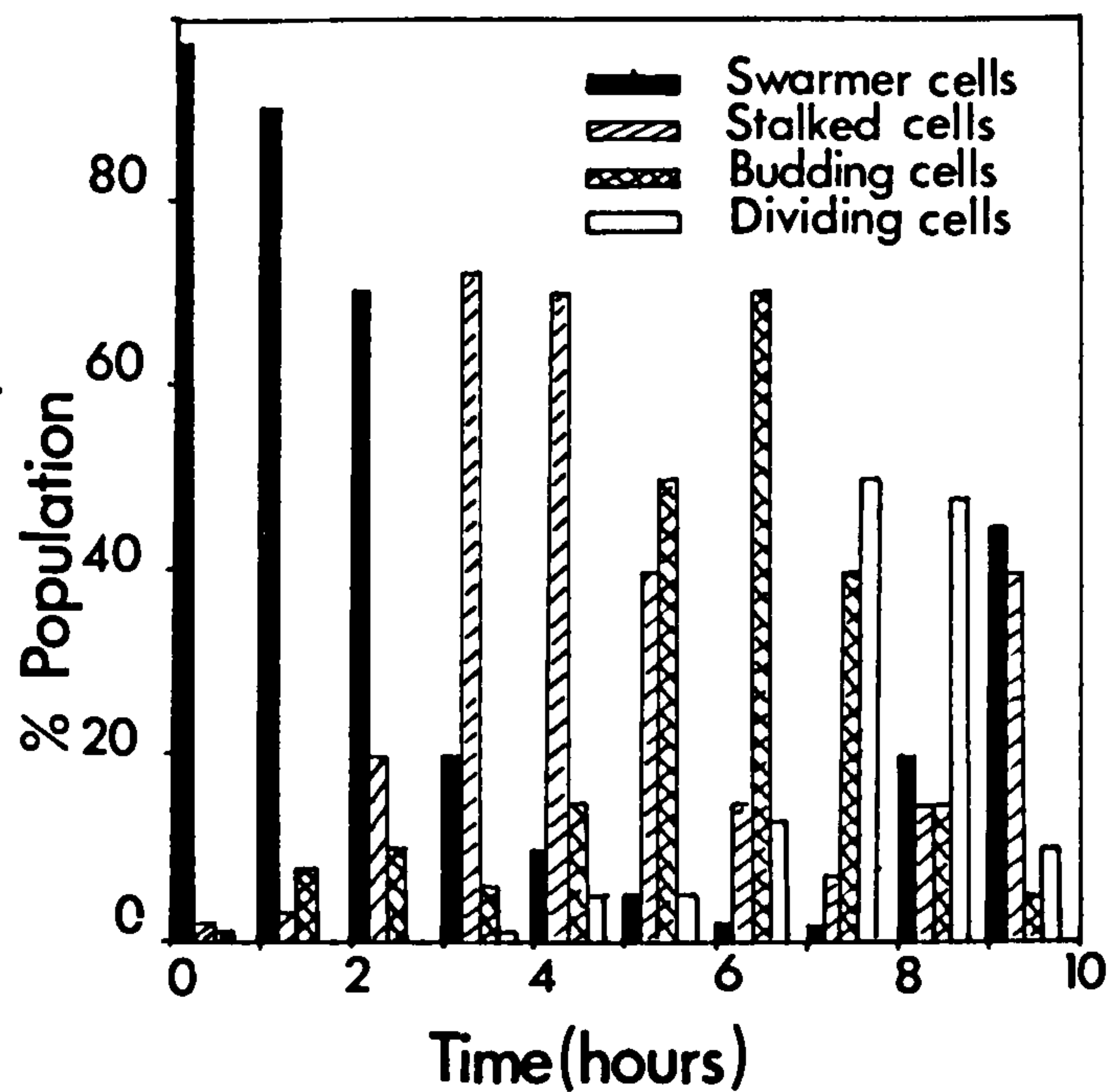


Fig. 3.14 Cell type variations in the synchronised cell population during the first generation. Swarmer cells rapidly developed stalks, resulting in stalked cells dominating the population after 3-4 h, and buds formed by 6 h. By 10 h the population was already out of synchrony.

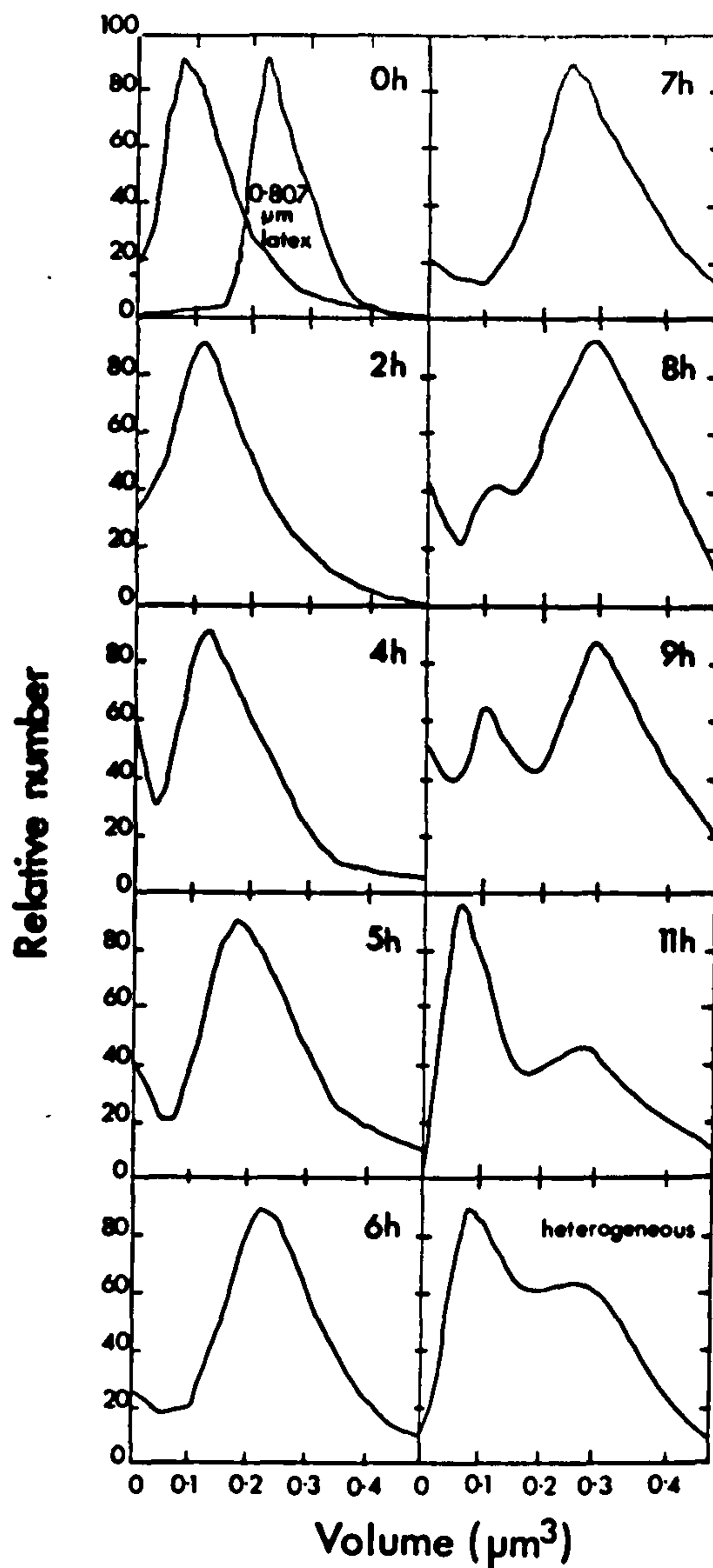


Fig. 3.15 Coulter counter study of a synchronised population of Hyphomicrobium, showing the variations in cell volumes. $0.807\ \mu\text{m}$ latex particles as reference. Peak at 0 h corresponds to immature swimmers, peak at 6 h and 7 h corresponds to budding cells. As cells divide (8h and 9h), two peaks are observed, one representing new swimmers, the other dividing cells.

immediately, shedding their flagella and increasing in cell volume (Fig. 3.15). The apparent 'levelling off' of growth between 3 and 5 hours (Fig. 3.13) coincides with stalk formation, suggesting that the stalks do not significantly contribute to the optical density, i.e. the cell biomass, of the culture (Fig. 3.13). The increase in cell numbers after 7 hours corresponds to the reappearance of swarmer cells and stalked cells (Fig. 3.13). This generation doubling is very distinct, however the second generation cells were already out of synchrony due to the inequality of the mother and daughter cell cycles, caused by the long swarmer cell maturation.

A microscopic examination and count of 500 cells in hourly samples revealed that the appearance and disappearance of different cell types was quite marked (Fig. 3.14). Comparing this to observations using the Coulter counter demonstrated that the peak observed in the trace at 0 hours corresponded to the swarmer cells (Fig. 3.15). As the cells matured, this peak shifted to the right (6 hours) until by 8 hours there were two peaks, one corresponding to the new swarmer cells, and the second, broader peak, corresponding to the mother cells in various stages of bud development.

(b) Life cycle and morphology

Hyphomicrobium appears as an ovoid cell of width 0.5-0.75 μm and of length, which is affected by age and growth conditions, of 0.5-3.0 μm . The stalks have a diameter of 0.2-0.3 μm , but their length varies considerably during growth (Figs. 3.16, 3.17, 3.18). The stalks branch and bifurcate in all stages, but this is more evident in older cultures (Rullman, 1897; Stutzer and Hartleb, 1898; Kingma -Boltjes, 1936; Zavarzin, 1960; Hirsch and Conti; 1964a, Geitler, 1965). In some strains a holdfast is evident on the cell body, opposite the stalk, resulting in characteristic rosette formation (Zavarzin, 1960) (Fig. 3.19). The stalked cells frequently contain one or more highly refractile granules of poly- β hydroxybutyrate (PHB) which are normally observed in mature

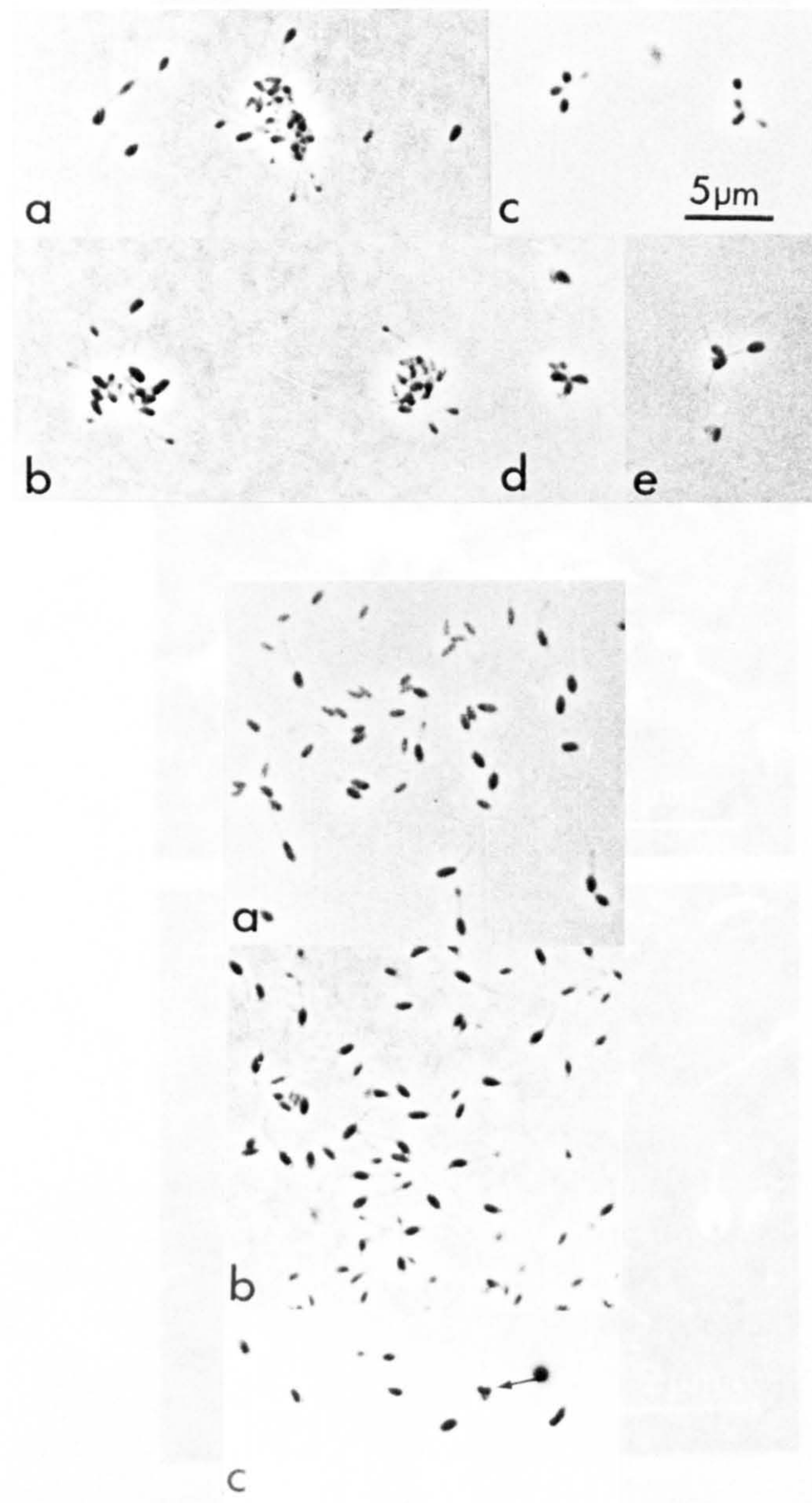


Fig. 3.16 Light micrographs of Hyphomicrobium culture. Top photomicrograph shows the culture in stationary phase, with cells forming aggregates, probably by mucilagenous adhesions rather than by holdfasts, whereas the lower photomicrograph shows the culture in exponential phase; a lobed cell is arrowed.

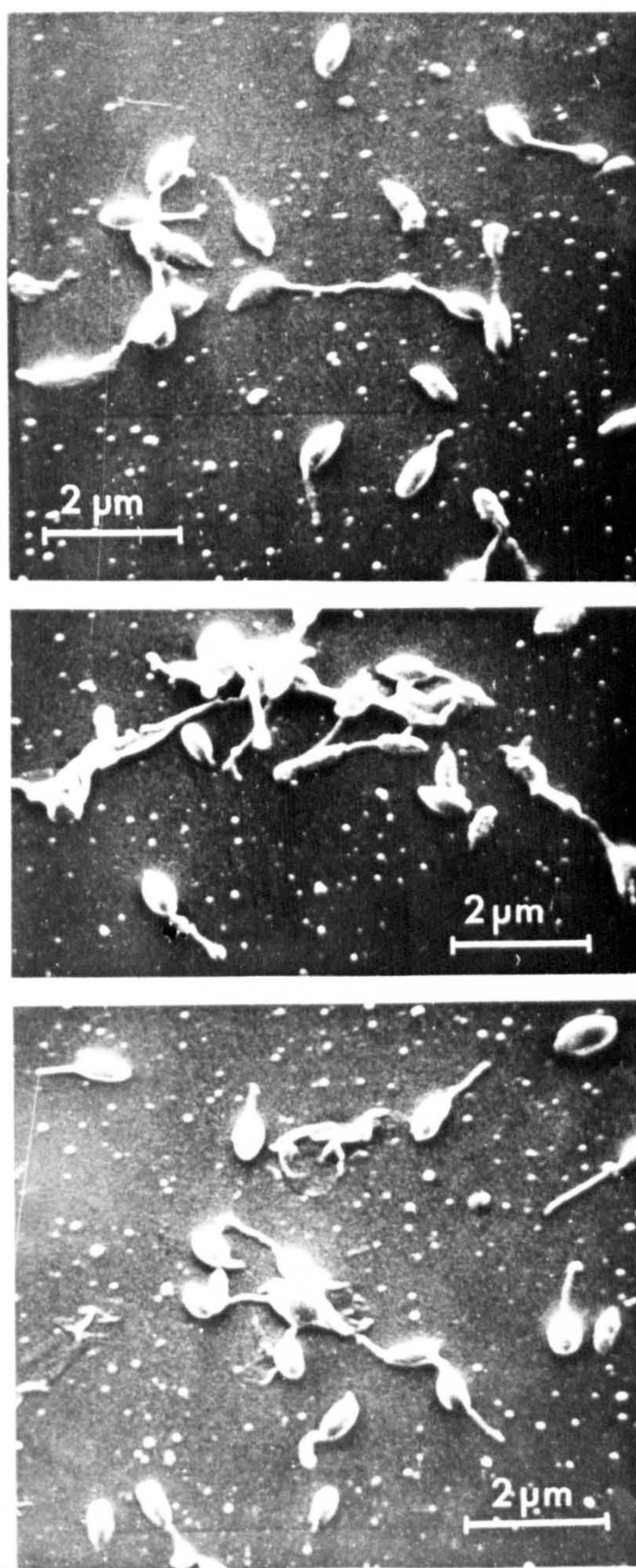


Fig. 3.17 Scanning electron micrographs of *Hyphomicrobium*, using glass coverslips as the supportive structure, showing the general relief of these cells. Critical point drying prevents the cells collapsing, as they are dried on to the support.

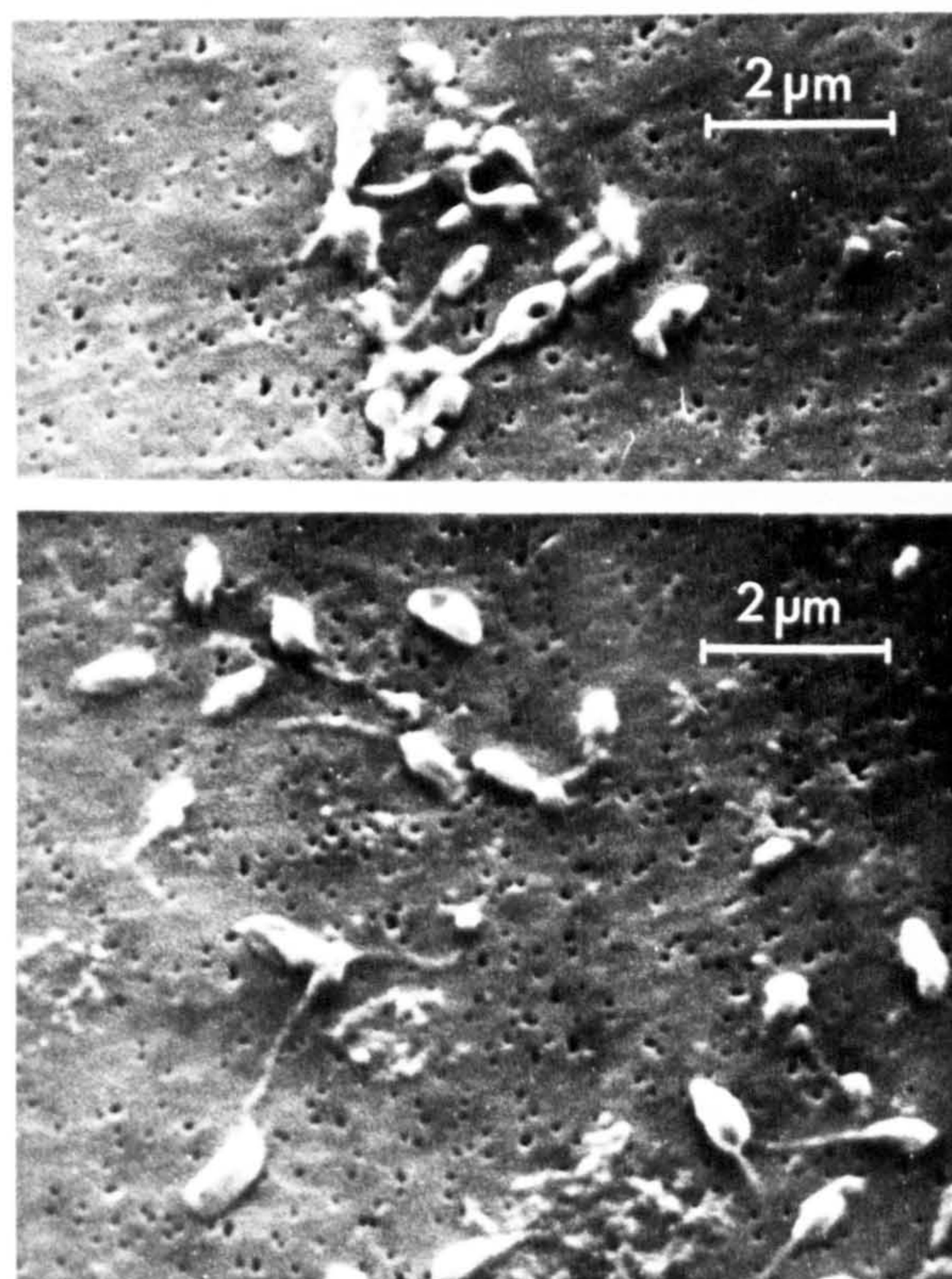


Fig. 3.18 Scanning electron micrographs of Hyphomicrobium using nucleopore membranes. Heterogeneous population illustrates various cell types. Membranes floated well on culture surfaces for indefinite periods of time, however their pore size, relative to the bacteria, masked morphological detail.

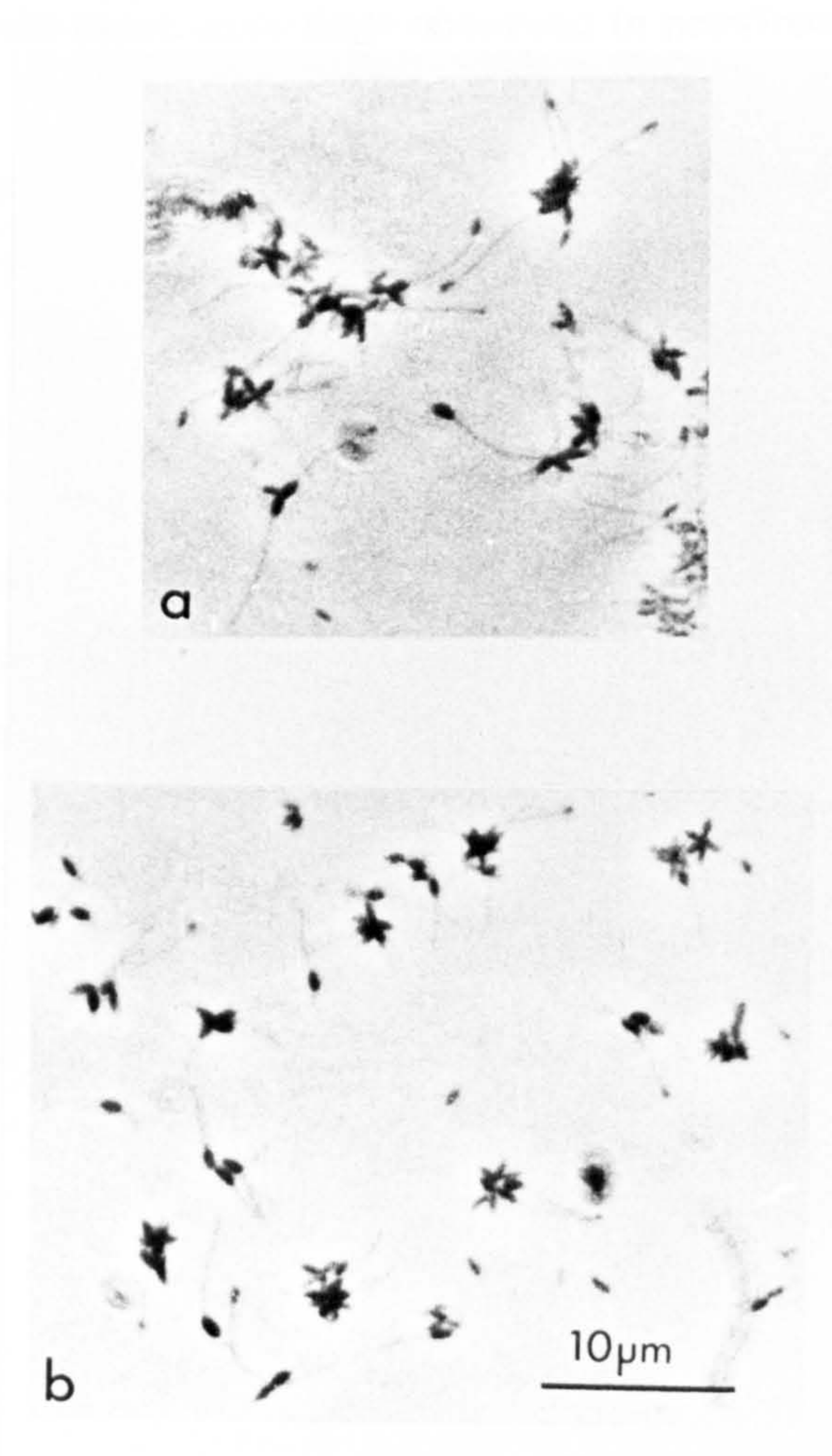


Fig. 3.19 Rosette formations in *Hyphomicrobium* cultures. The holdfast is normally located at the pole, free from bearing the stalk. Consequently aggregates have stalks radiating outwards, the buds unrestricted for dispersal (cf. *Caulobacter*, Poindexter, 1964).

cells in late exponential or stationary phase of growth (Doudoroff and Stanier, 1959; Parnas and Cohen, 1976). Reproduction occurs by the formation of a bud at the stalk tip (Blackman and Weiner, 1975) during the maturation of which a single subpolar flagellum is formed, although up to three have been observed in previous studies (Hirsch and Conti, 1964a; Harder and Attwood, 1978), and by some workers they have been observed polarly as well as subpolarly (Kingma-Boltjes, 1936; Zavarzin, 1960; Guillard and Watson, 1962; Leifson, 1964; Hirsch and Conti, 1964; Takada, 1975). After a period of time the bud breaks off from the stalk, vigorous movements being thought to cause the detachment, so becoming an active swarmer cell. Eventually the swarmer cell sheds its flagellum, matures and develops a filament from one of the poles, to become a reproductive mother cell and so repeat the cell cycle. Mother cells produce new buds sequentially from the ends of pre-existing stalks (cf. Hirsch and Jones, 1968) (Fig. 3.1). The life cycle was determined from light and electron microscope studies (see slide culture results, Figs. 3.21, 3.22, 3.23).

The life cycle of Hyphomicrobium has been shown in this thesis and by many other workers (Section 3.1) to be the formation of a bud at the tip of a stalk, with asymmetric division yielding a motile swarmer cell and a stalked mother cell (Fig. 3.20). Previous workers (Hirsch and Conti, 1964a; Hirsch, 1974) have stated that the daughter cell produces its stalk at the pole distal to its point of attachment to the mother cell. The presence of double stalked cells has also been noted (Zavarzin, 1960; Leifson, 1964; Hirsch and Conti, 1964a), but no mention was made of the capability of these double stalked cells to produce viable daughter cells (Hirsch, 1974). Slide culture studies were therefore carried out, to try and resolve in detail the developmental cell cycle when there is more than one stalk present, and to determine the orientation of these daughter cells with respect to the mother cell. As growth conditions can drastically alter the morphology of Hyphomicrobium, the cultures used were heterogeneous or synchronised

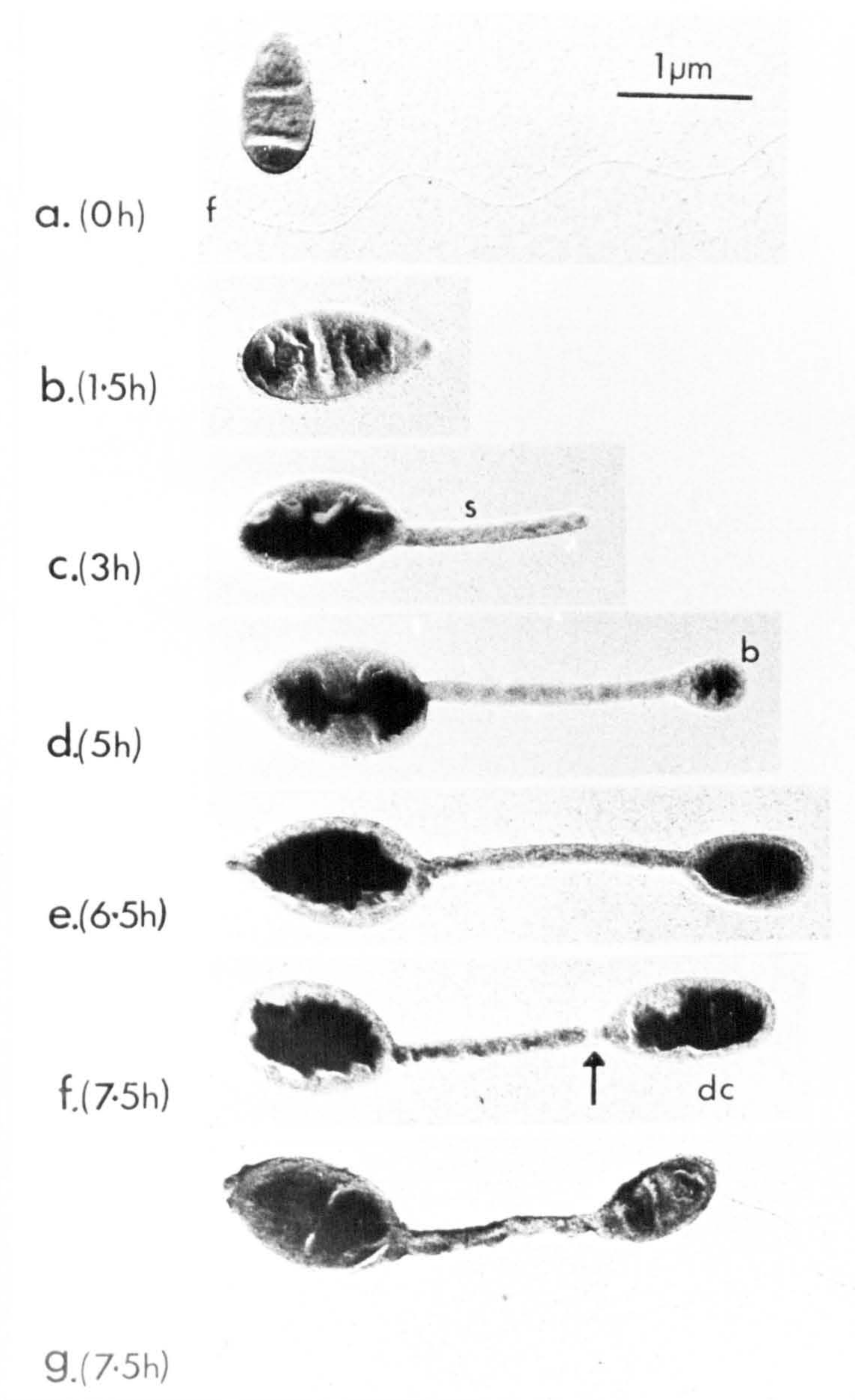


Fig. 3.20 Electron micrographs of *Hyphomicrobium*, synchronised population. At 0 h flagellum (f) is evident; stalk (s) and bud(b) develop, and by 7.5 h the daughter cell (d.c) is constricted from the mother cell.

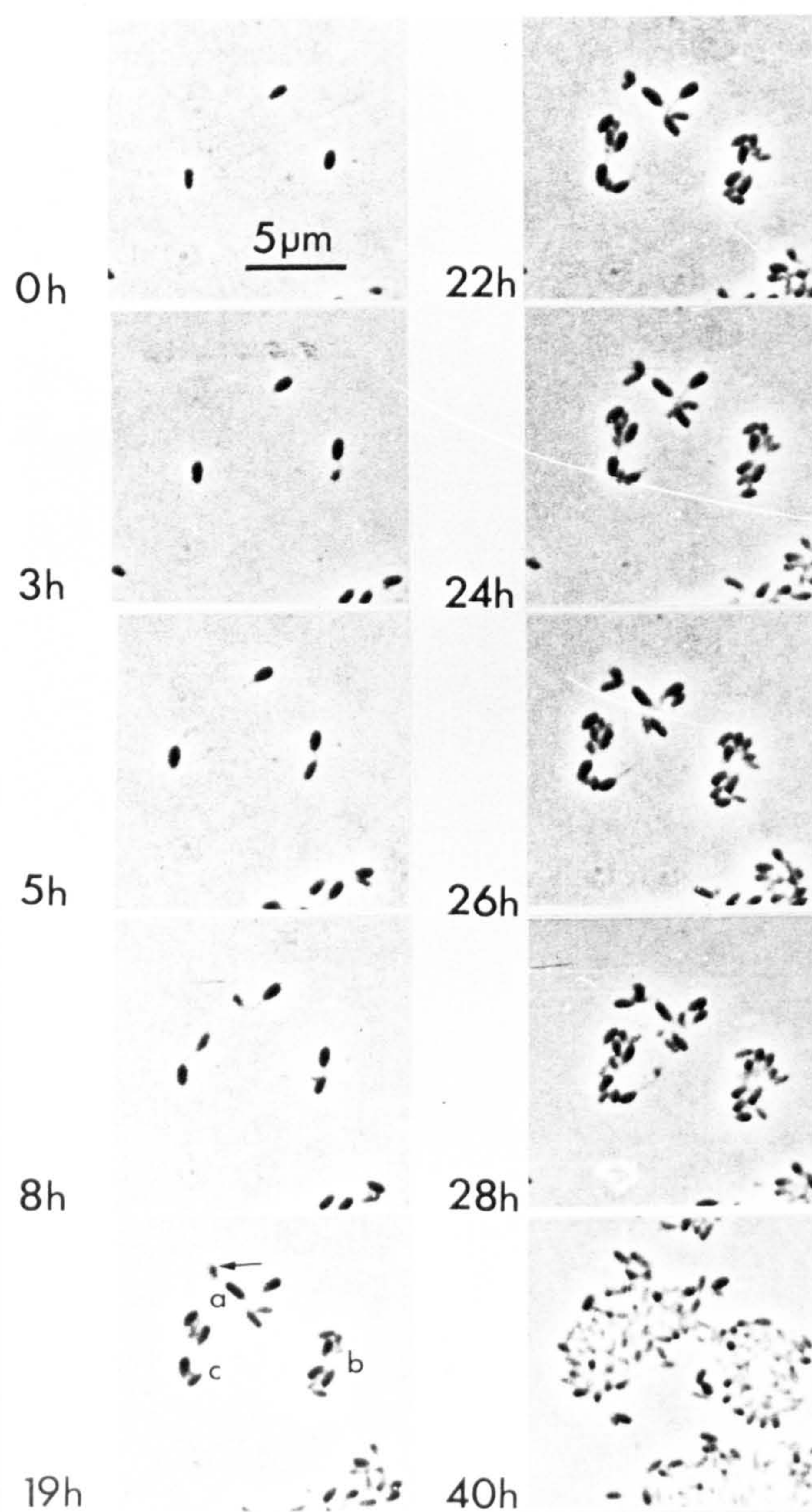


Fig. 3.21 Slide culture of synchronised Hyphomicrobium showing the development cycles. In frame 19 h, cluster (a) a daughter cell is developing from the distal pole (arrowed), in cluster (b) stalks develop from the apical poles, whereas in cluster (c) first generation buds are produced from both apical and distal poles.

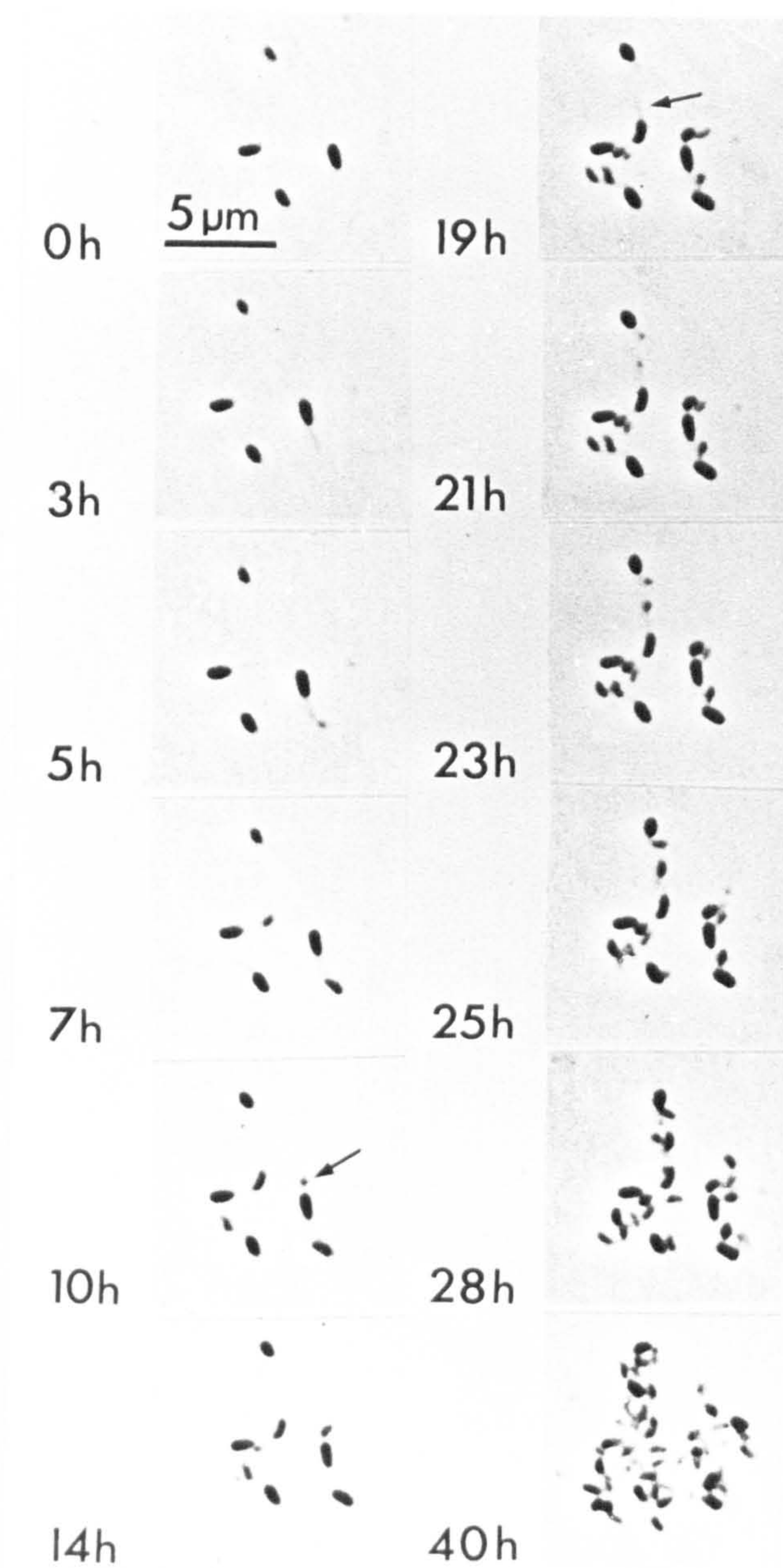


Fig. 3.22 Slide culture of synchronised *Hyphomicrobium*, showing the development of buds, when cells are bistalked. Frame 10 h. shows bistalked cell (arrowed) developing bud from second stalk. Frame 19 h. similarly shows that only one stalk develops a bud at any one time.

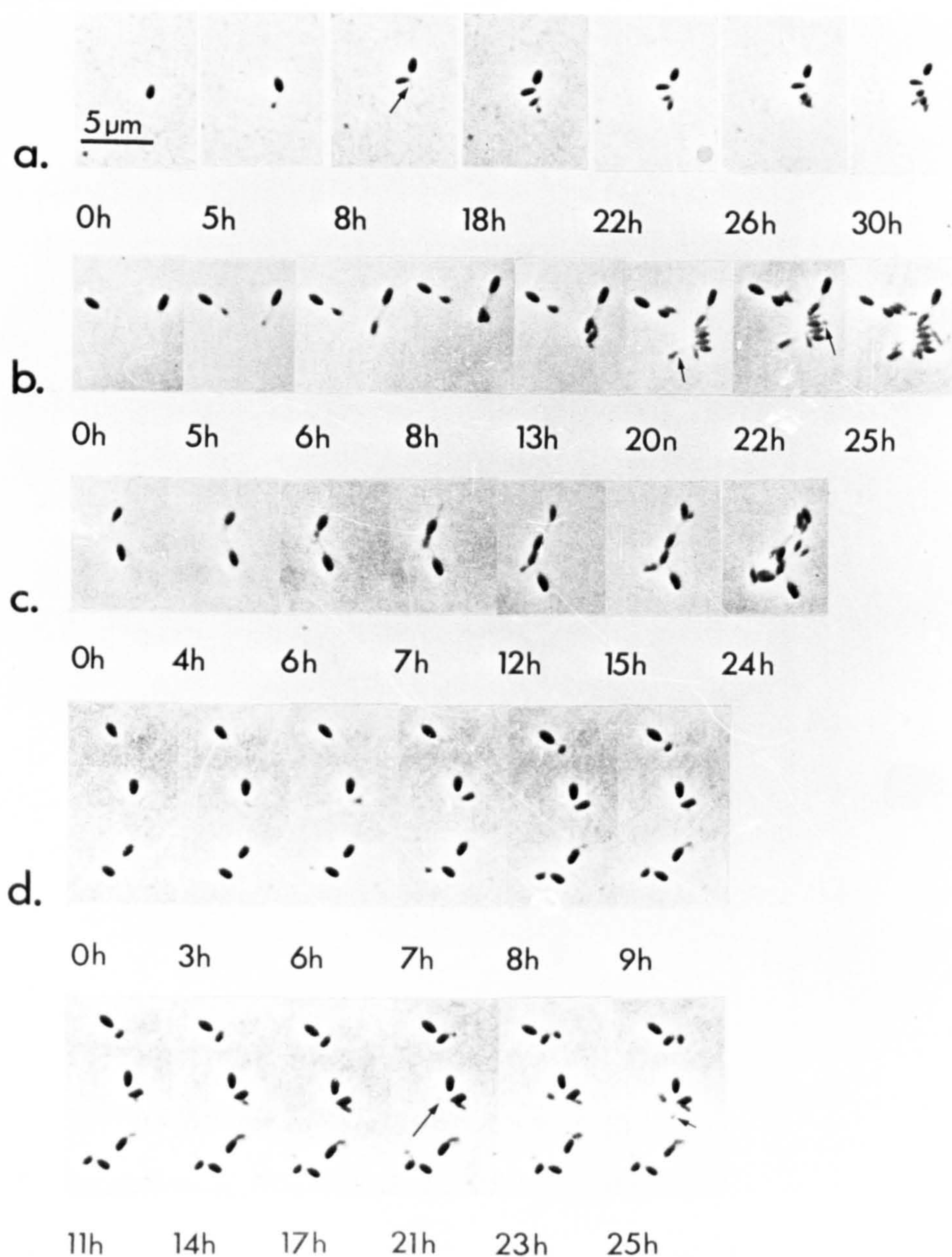


Fig. 3.23 Slide cultures of synchronised *Hyphomicrobium*. (a) and (b) illustrate the obligate period of stalk synthesis as each bud is developed; (c) and (d) show how the cells can develop buds from one or two stalks, and the buds can then develop stalks from the same or opposite pole from their point of attachment to the mother cell (d).

populations, grown under optimal conditions as described in Section 3.II.3, which included minimising light disturbance from the microscope beam by the use of a green filter (which reduced heat given off by the light bulb).

Fig. 3.21 shows the classical development of a synchronised population of swarmer cells as previously described (Section 3.I). First generation buds were formed by 8 hours. These then developed stalks at the apical or distal pole, with respect to their attachment to the mother cell, i.e. the apical pole of the daughter cell was attached to the stalk of the mother cell. The orientation remained constant as the cells were immobilised on the agar. In frame 19h (Fig. 3.21) it can be seen that in cell cluster (a) a daughter cell is developing a stalk from the distal pole (arrowed), in cell cluster (b) stalks develop from apical poles, whilst in cell cluster (c) first generation daughter cells are producing stalks from the apical and distal poles. Thus the development choice of cell pole for the stalk appears random, although distal stalk development is most common. Figs. 3.22 and 3.23 show typical slide culture studies of Hyphomicrobium with "staggered" (daughter cell produces bud at opposite pole to its attachment) and "zig-zag" (daughter cell produces bud at same pole to its attachment) colony development and Fig. 3.24 depicts the various ways the Hyphomicrobium cell can develop. Hyphomicrobium therefore resembles Rhodomicrobium in its ability to produce stalks from either pole (Dow et al., 1976). A possible controlling factor which determines from which pole the stalk develops could be the immediate environment about that particular cell.

When the Hyphomicrobium cell developed two stalks, a bud was only produced on one stalk at a time (Fig. 3.22). Again, in this respect it resembles Rhodomicrobium (Dow. et al., 1976) and the controlling mechanisms may be similar. Whittenbury and Dow (1977) have shown that with Rhodomicrobium there is always a period of stalk synthesis between sequential bud formation. This is clearly seen in Hyphomicrobium (Fig. 3.23 a and b) where stalk synthesis occurs from the same pole (arrowed) throughout the period of study. From

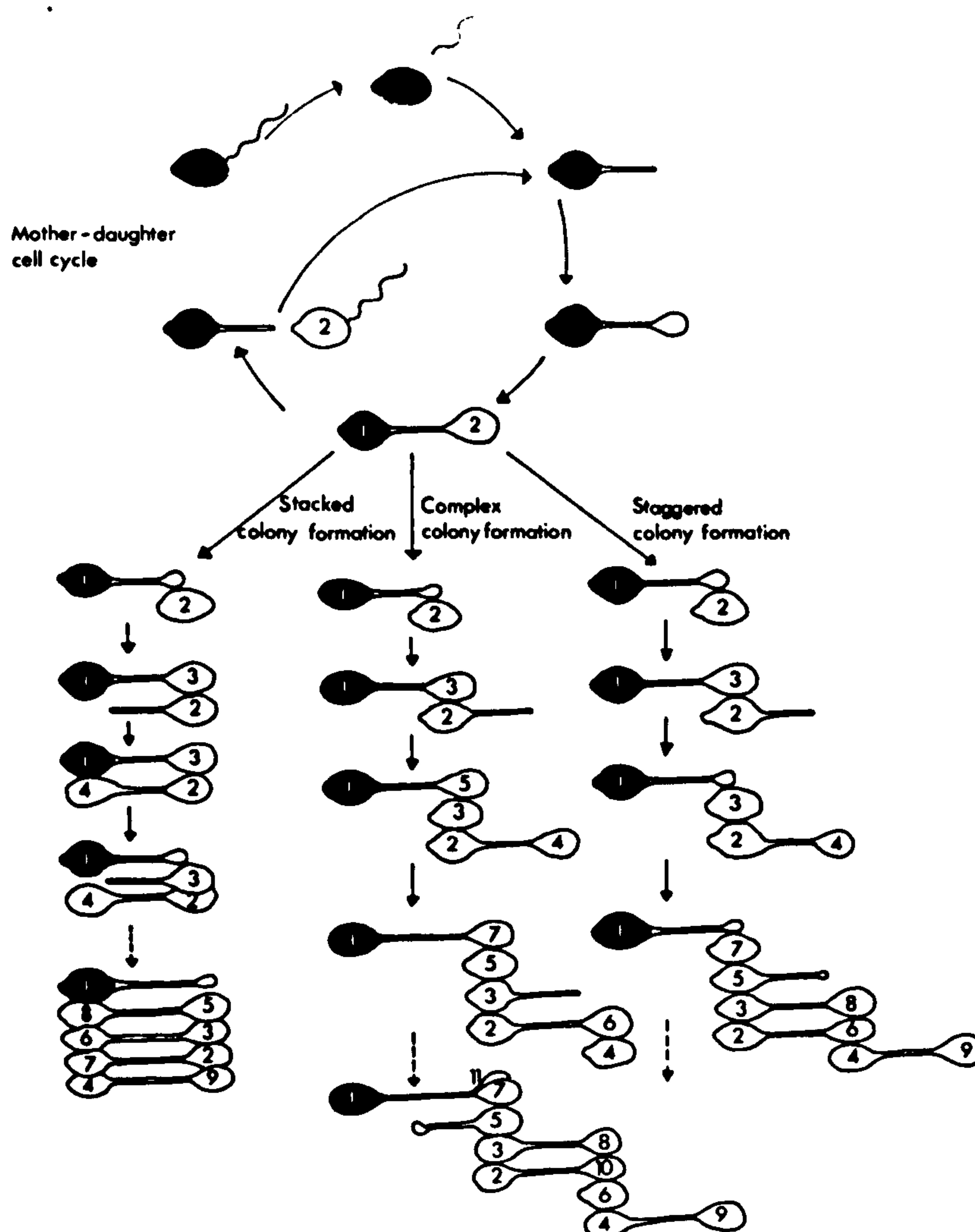


Fig. 3.24 Summary of colony formation in slide culture, illustrating stacked or 'zig-zag' colony formation, 'staggered' colony formation and 'complex' colony formation. Numbers represent order in which buds are formed from the mother cell and subsequent daughter cells.

some studies, mother cells could be shown to give rise to at least seven buds, agreeing with previous workers (Kingma-Boltjes, 1936; Mevius, 1953) (Fig. 3.23 a and b), with a compulsory increase in stalk length after each bud development, giving rise to the staggered pattern of buds in the colony. The number of daughter cells arising from a single mother cell appeared to be limited, possibly due to the ageing of the mother cell or some other undetermined factor. A similar situation occurs in Rhodomicrobium (Dow et al., 1976) with the mother cell only being capable of giving rise to four daughter cells or four exospores. Fig. 3.24 summarises microcolony development in Hyphomicrobium on solid medium, however this is an artificial environment, and consequently may not completely reflect the life cycle in the natural environment. Whittenbury and Dow (1977) have suggested that a reason for this stalk synthesis is that the DNA destined for the daughter cell is attached to the cytoplasmic membrane at a potential growing point of the cell. The DNA is drawn into the bud by the cytoplasmic membrane. The switch from filament to bud synthesis is preceded by division of the growth point and of the DNA attachment site. One growth point remains active and gives rise to the daughter cell, whilst the other is dormant. Reactivation of this growth site occurs on separation of the mother and daughter cell. The formation of the second daughter cell is preceded by further stalk synthesis which again facilitates segregation of the DNA attachment sites (Fig. 3.23 a and b). With Rhodomicrobium, a cross wall is laid down between mother and daughter cells (Dow et al., 1976), so that they are essentially independent, enabling both to produce daughter cells. With Hyphomicrobium, separation of the cells is by binary fission. Occasionally cell division does not occur, and a chain of cells is formed (Section 3.III.4). It is not evident whether cells comprising the chain are physiologically independent or if continuity is maintained. There is no plug formation as is found in Rhodomicrobium (Conti and Hirsch, 1965).

(c) Batch and continuous culture - population dynamics ?Introduction1. Batch culture

Increase in biomass during growth of budding bacteria has been measured in terms of the dry weight, protein or DNA content of the cultures (Hirsch and Conti, 1964b). Such measurements do not provide an adequate description of the morphological changes which occur during the growth of budding bacteria in batch culture. The work of Bauld and Marshall (1971), Bauld and Tyler (1971) and Bauld, Tyler and Marshall (1971) overcame this problem to a certain extent by introducing the terms 'colony-forming unit' (CFU) which described a metabolic unit capable of giving rise to a single colony on an appropriate solid medium, and 'cell number' (CN) which referred to the varying number of cells which may comprise a single cell ($N = 1$) or a multi-cellular unit containing two or more cells interconnected by stalks. A quantitative measurement of the distribution of the various morphological forms of Hyphomicrobium was given by the 'population index' (PI) where

$$PI = \frac{\text{number of CFU's present}}{\text{CN's comprising these CFU's}} = \frac{\sum CFU}{\sum CN}$$

With this model they studied cultures for up to 400 hours in batch culture and showed that a swarmer cell population rapidly becomes a mixture of colonial forms, i.e. CFU varied from 1 to 7. By using these criteria, they showed that there could be increases in the cell population, with little alteration to the total biomass. These measurements provided a quantitative estimate of the distribution of the various morphological forms during the growth of Hyphomicrobium T37. There were, however, restrictions to their model in that firstly there was no consideration given to how the individual cell types, i.e. swarmer, stalked and budding cells, varied within the total population throughout the time course, and secondly the system was only considered under batch conditions, and so no correlation could be made between cell morphology and growth rate.

A disadvantage of studying populations of cells in batch culture is that the organism has to be grown at nutrient concentrations that are much higher than those occurring in environments such as freshwater. In batch

culture, microorganisms grow at a maximal rate in the presence of an excess of all nutrients, a situation rarely, if ever, encountered in nature. Only at the end of the phase of exponential growth will a component of the medium become growth-limiting; however, this limitation will be only for a brief period of time and subject to continuous changes in the chemical composition of the culture medium (Veldkamp, 1976).

2. Continuous culture

Microbial growth under nutrient limitation, with strictly controlled parameters, can be achieved by using a chemostat, as described in Section 3.II.23. In continuous culture, fresh growth medium is introduced continuously into the culture vessel, culture liquid containing microorganisms being simultaneously removed at the same rate. In the chemostat, cells grow at a submaximal rate as they do in their natural environment, and these cells can be grown in steady states at any one of a whole range of growth rates. The only factor determining a particular growth rate is the concentration of the growth limiting substrate, which is determined by the dilution rate. A microbial culture in the chemostat grows under substrate limitation which is the natural situation in the environment; however, the chemostat does not mimic a natural environment in any way. In natural environments growth conditions change continually, and in evolution selection has favoured those organisms that could cope with these changes: steady state conditions as occur in the chemostat rarely occur in nature. A further restriction of the chemostat is that it only allows studies of growing cells (Veldkamp, 1976).

The chemostat is ideal for studying the properties of an organism as a function of growth rate, and offers many possibilities for the detailed study of single microbial species and the interactions between different microorganisms and their environment (Wilkinson et al., 1973).

Studies were therefore undertaken to determine how the cell types which contribute to the Hyphomicrobium cell cycle varied in the total population, in batch and continuous culture. Stalk length was measured

along with overall cell volume to determine if there was any correlation between these parameters, and the growth rates measured.

Results and Discussion

Batch culture studies

Hyphomicrobium isolates I and X were monitored throughout the growth cycle, by spectrophotometry, protein determination, viable cell counts, Coulter counting and microscopical observations. The first three methods are conventional, showing classical growth curves with lag, exponential and stationary phases. Only by microscopical observation and particle sizing could the population be studied with respect to the constituent cell types.

Swarmer cells Considering the culture as a whole, Coulter counter traces (Fig. 3.25) and microscopical observations (Fig. 3.26) showed that initially a large component of the population was represented as a peak corresponding to approximately $0.05 \mu\text{m}^3$, the swarmer cell stage in the life cycle. As the culture developed, the swarmer cell number varied considerably, reaching a minimum between 30 and 40 hours (Fig. 3.26). Particle sizing indicated that these swarmers were larger in volume than those observed at inoculation (stationary phase) (Fig. 3.27). Fig. 3.28 shows that there is an inverse correlation between swarmer cells and budding cells throughout the growth cycle, with the ratio of swarmer cells to budding cells against time reaching a minimum during exponential growth. As the culture reached stationary phase, the peak shift was to the smaller cell volume again, corresponding to increased numbers of swarmer and stalked cells (Fig. 3.26). These variations in the swarmer cell population were not reflected in the conventional methods for monitoring the growth cycle (Fig. 3.29).

Stalked cells Stalked cells were monitored by microscopy, enabling their relative number in the population to be determined. Although the length of the stalk appeared to vary during the growth cycle (Fig. 3.27) this could not be detected by the Coulter counter as any differences in stalk volume appeared negligible compared to cell volume changes.

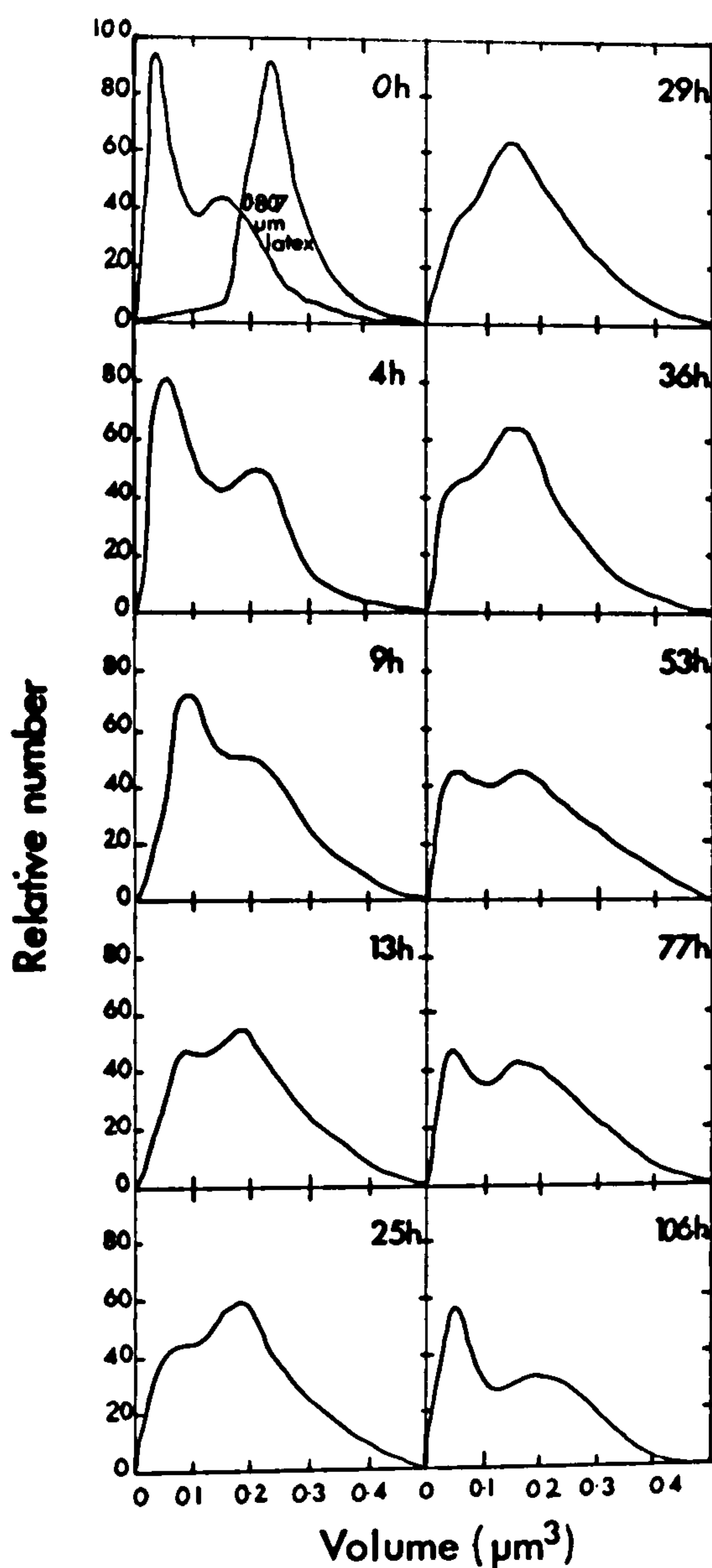


Fig. 3.25 Coulter counter traces of *Hyphomicrobium* through a batch culture. 0 h represents inoculation of the culture, the peak representing swarmer cells blocked in development, whereas by 77 h the culture was in stationary phase and the peak had returned as swarmer cells again were blocked. During exponential growth the peak shift was to the right, representing budding cells. (Settings: 1/aperture current = $\frac{1}{2}$, 1/amplification = $\frac{1}{2}$). 0.807 μm latex as standard.

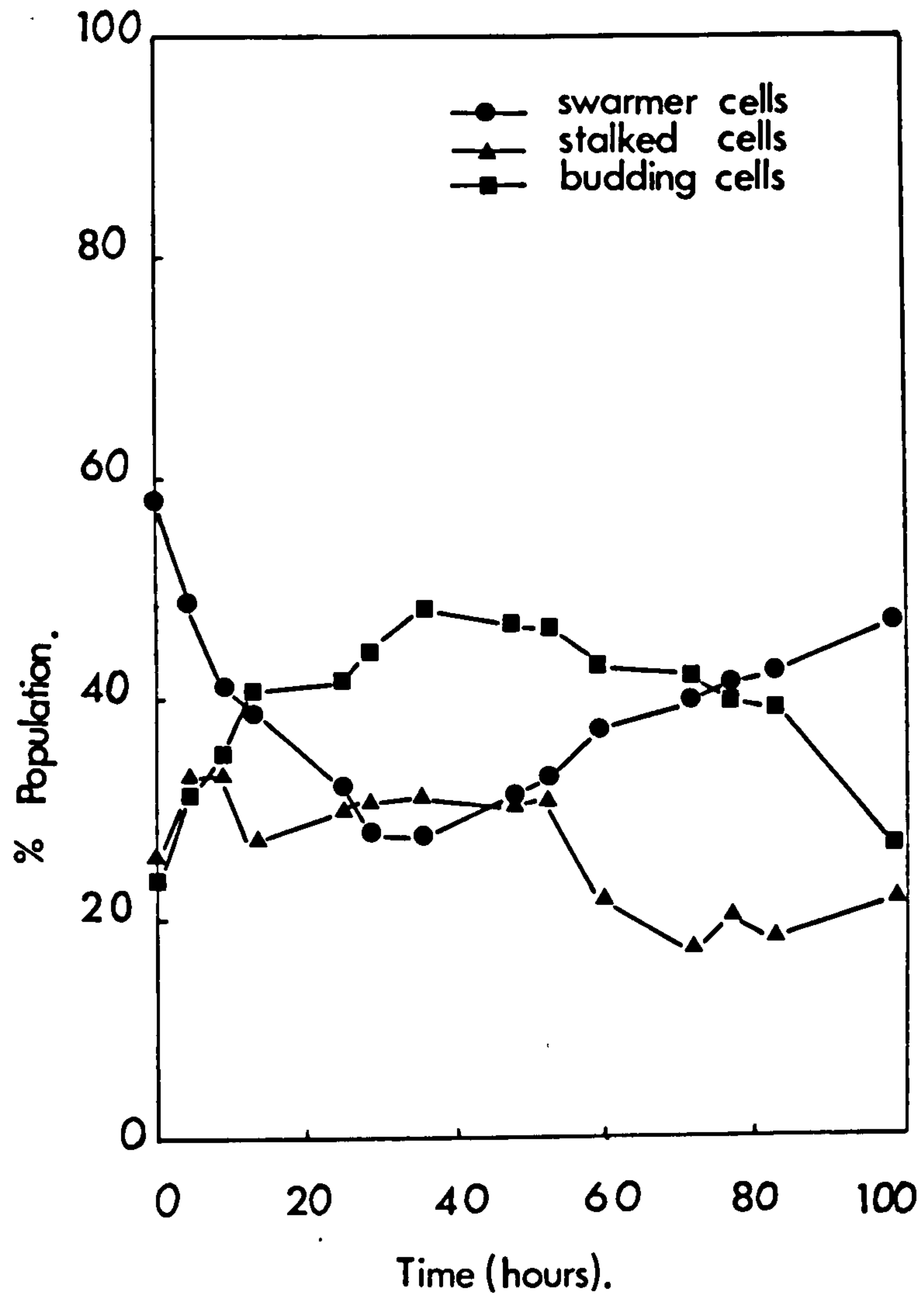


Fig. 3.26 Variations in cell types in the population through the growth curve. Cell types represented as % of total population. Counts determined by microscopy. Stalked cell numbers remained relatively constant, however there appeared to be an inverse correlation between swarmer cells and budding cells during the growth of the culture.

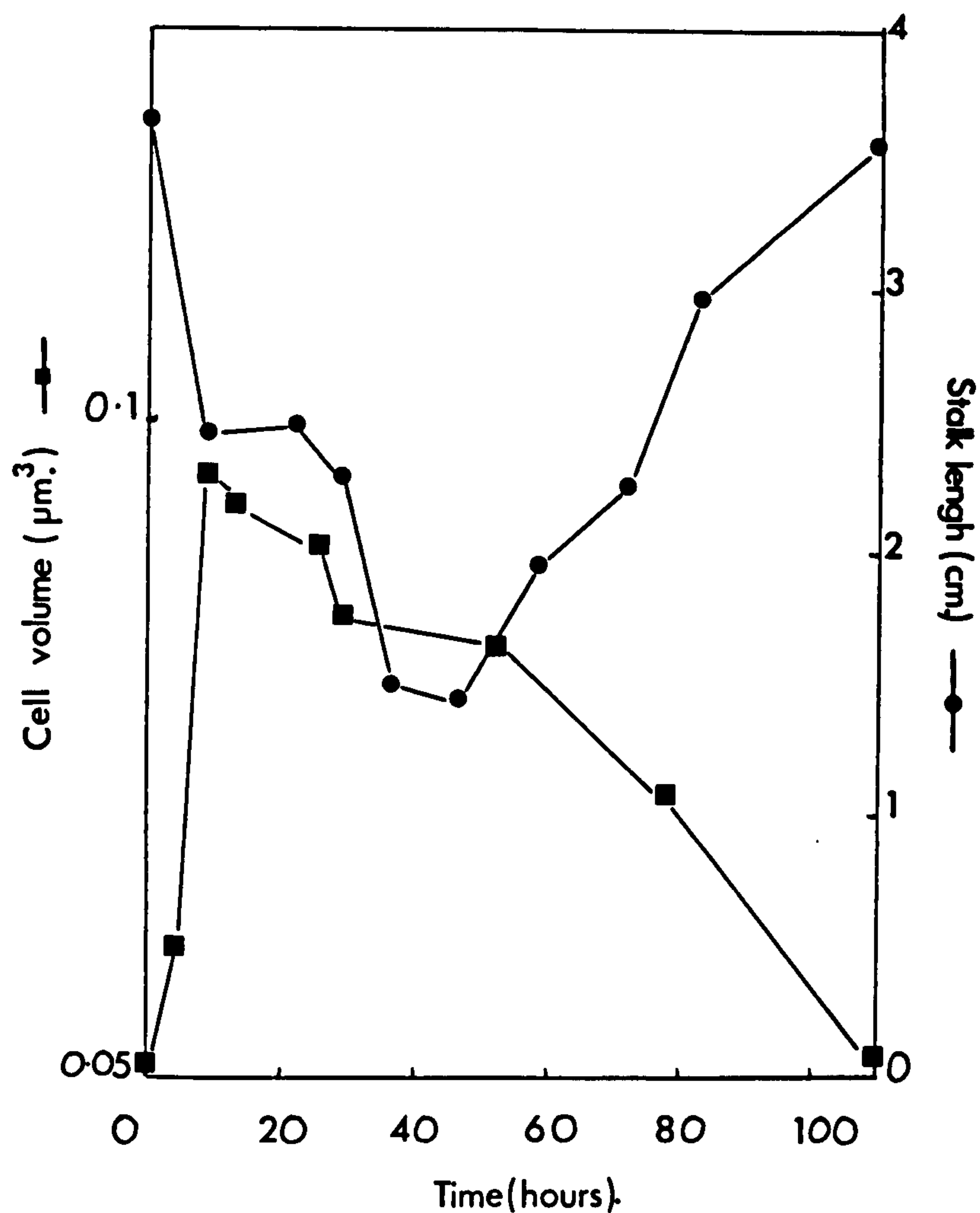


Fig. 3.27 Variations in cell volume of swarmer cells (—■—), and length of the stalk in stalked and budding cells (—●—) during the batch culture development, determined by Coulter counter and electron microscopy.

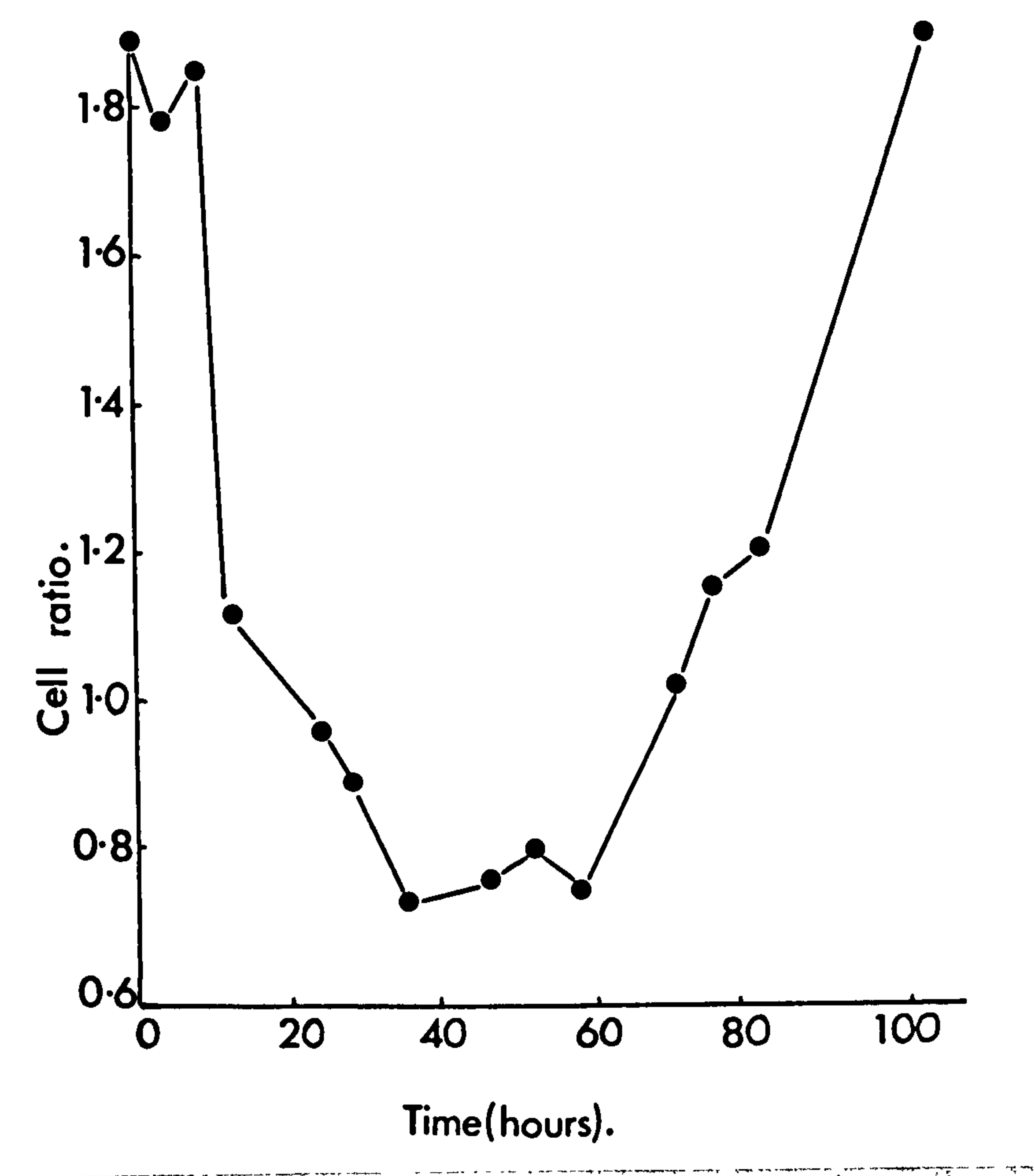


Fig. 3.28 Variations in ratio of swarmer cells to budding cells in the cell population, as the batch culture developed. Cell numbers determined by analysis of Coulter traces.

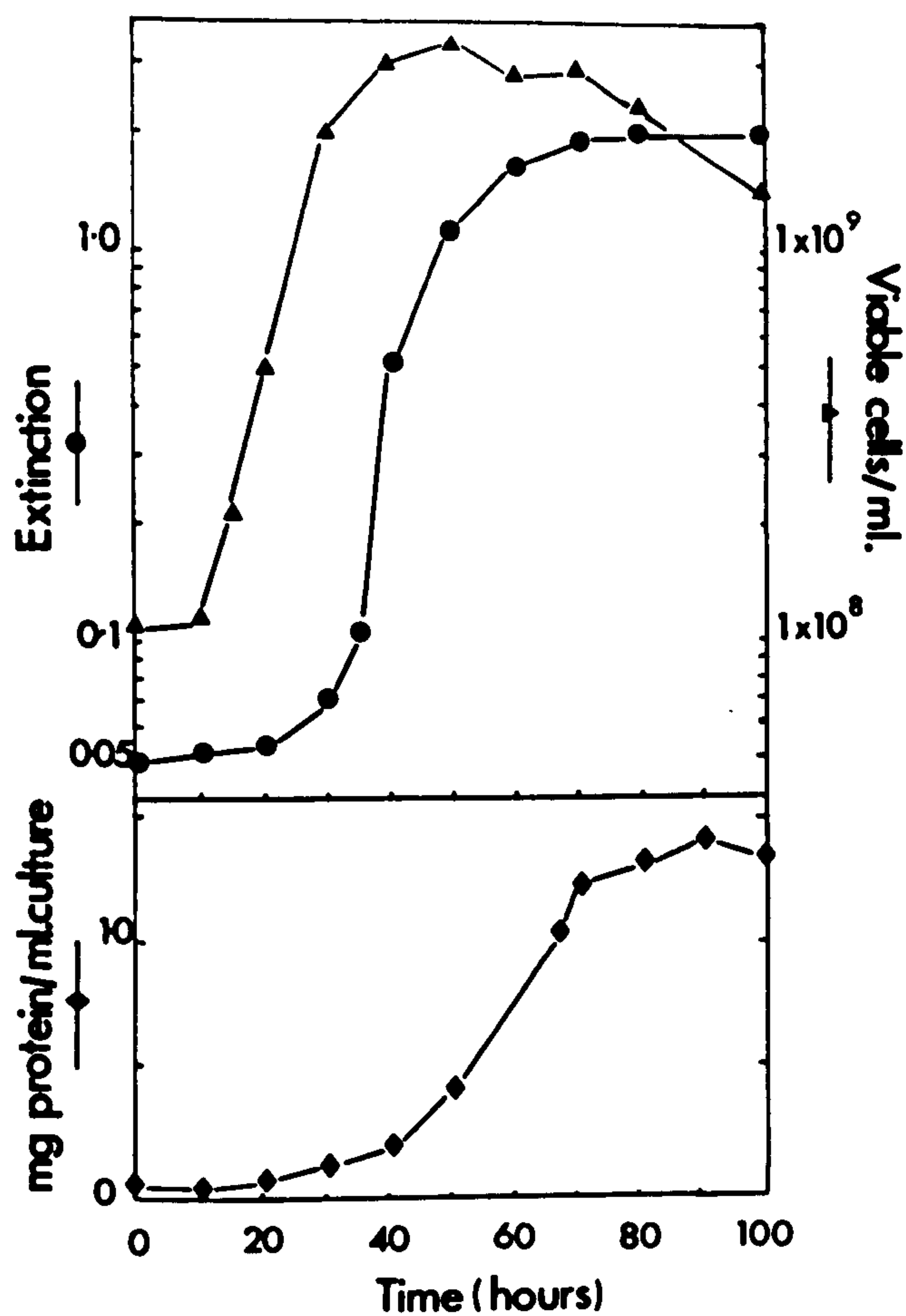


Fig. 3.29 Viable cells, determined by plate counting, extinction measurements and protein determinations during a batch culture study. Each demonstrated a lag period in the growth curve, but did not show how the population varied in its member components over this time course.

Budding cells The budding cell reflected morphological changes which also occurred in stalked cells, with respect to stalk length and volume of the cell body. Budding cells from the stationary phase inoculum had slightly elongated cell bodies and stalks between 3-4 μm in length (Fig. 3.30). It was very common, initially, to observe many of the budding cells with small swellings at the tip of the stalk, few mature buds were observed, and no cells in the process of cell division were noted (Fig. 3.26). During this early stage of the growth cycle, prior to exponential growth, the budding cells appeared elongated, however once into exponential growth, cell volume increased and the cell bodies of the budding cells became very rounded and the stalks became shorter (Fig. 3.30) reflecting the growth rate (Harder, personal communication). During the exponential phase budding cells increased in number, corresponding to the second peak at $0.2 \mu\text{m}^3$ (Fig. 3.25), however as the growth cycle approached stationary phase their numbers again dropped, the peak decreased, shifting to the left (Fig. 3.26) and the cell bodies and stalks became elongated. (Fig. 3.27).

Considering the population as a whole, once the culture was into the exponential phase of growth, swarmer cell numbers decreased and budding cells predominated. Budding cells were, however, rarely seen with mature buds, as with a rapid growth rate, no sooner was a bud formed at the stalk tip than it was constricted from the mother cell. This period of maximum cell division was observed as a combined increase in stalked and swarmer cells (Fig. 3.26). Thus it can be seen that throughout the growth cycle, the members of the cell population are constantly changing in predominance, and there appears to be some correlation between these variations and the growth rate throughout the cycle. It was interesting to note that throughout the batch growth cycle not only was there an inverse correlation between swarmer cells and budding cells against time (Fig. 3.28), but the ratios of swarmer cells to stalked cells and budding cells to stalked cells gave an inverse correlation (Fig. 3.31) suggesting that there is an inverse relationship between the swarmer cells and budding cells within the cell population.

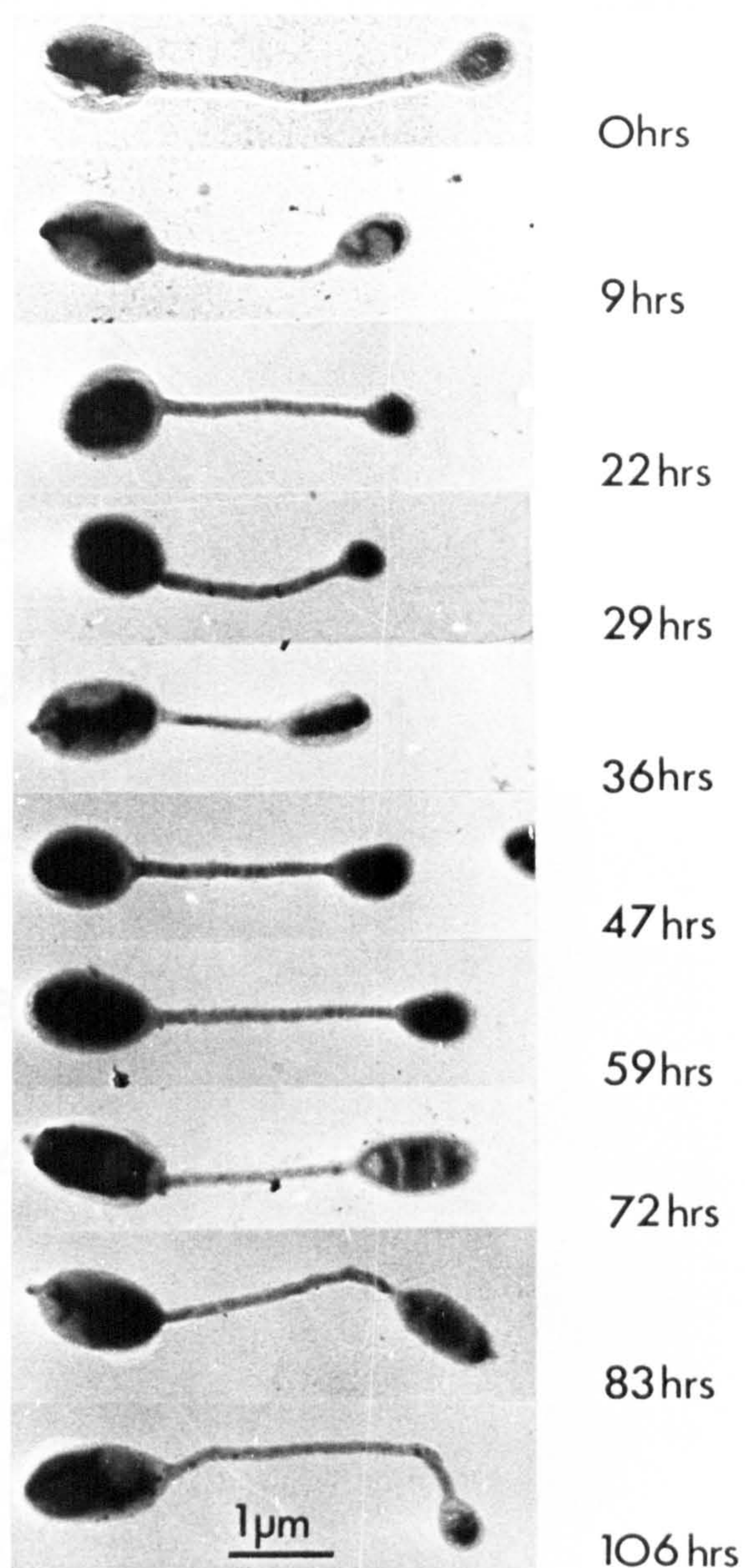


Fig. 3.30 Variations in stalk length and cell shape, as determined by electron microscopy, during the batch culture study. Cells rounded and stalks shortened during exponential growth. At inoculation and in stationary phase the stalks were elongated, and the surface area to cell volume ratio increased.

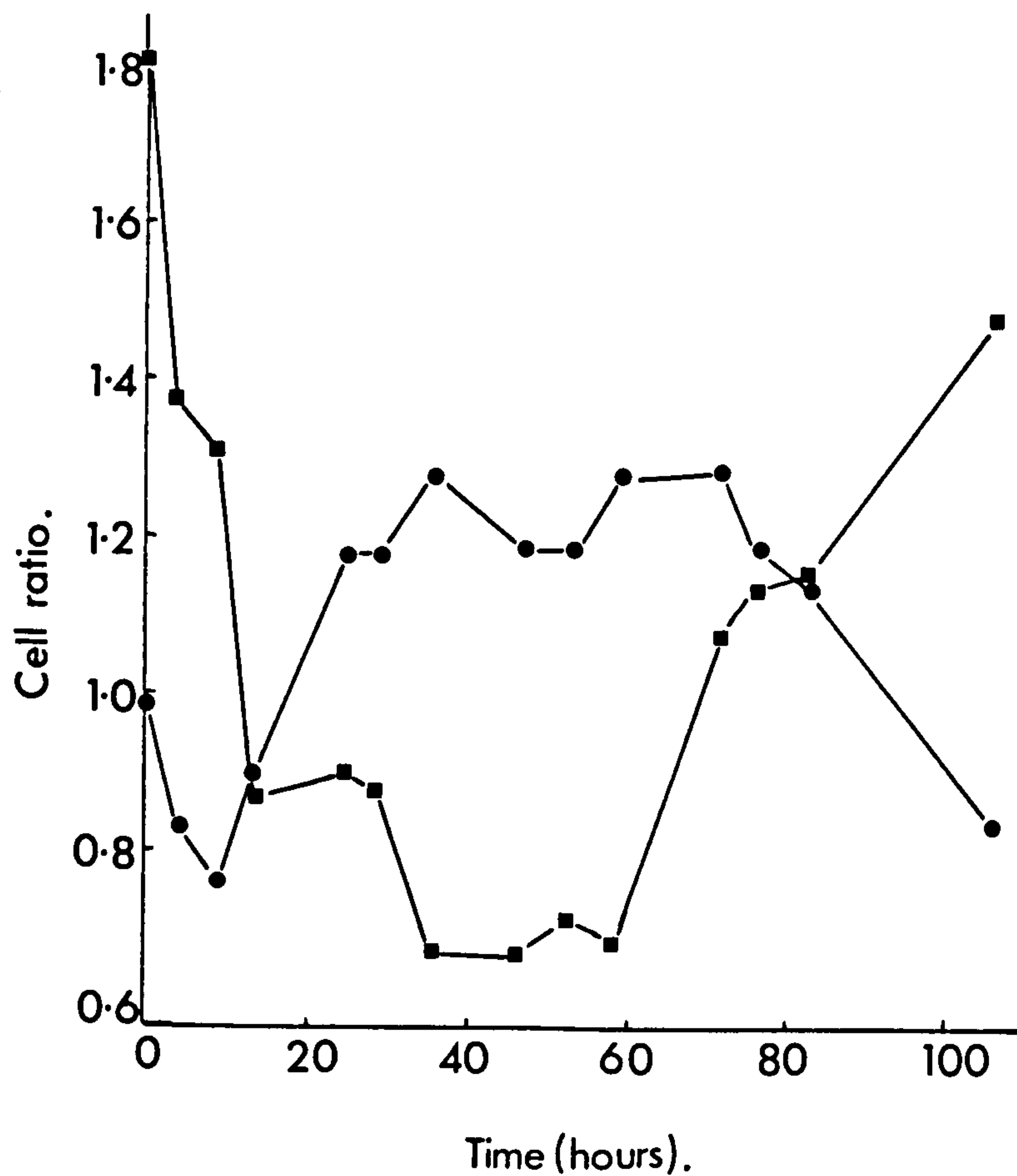


Fig. 3.31 Variations in cell ratios, swarmer to stalked cells (—■—), and budding to stalked cells (—●—) during the batch culture study. The inverse correlation reflects the growth rate under such conditions, i.e. during exponential growth reproduction is maximum and budding cells predominate, however in stationary phase cell survival is reflected by an increase in the number of swarmer cells.

Continuous culture studies

Preliminary studies were carried out on Hyphomicrobium in a 500 ml chemostat, in order to determine if these population variations were related to the growth rate. At low dilution rates ($D = 0.02 \text{ h}^{-1}$) the cells were limited for the carbon substrate, and consequently the cells grew poorly with a reduced cell volume, but the surface area to cell volume ratio increased (Harder, personal communication) compared to exponentially growing batch culture cells. Stalk length was also increased (Fig. 3.32), and multicellular arrays were not uncommon. At dilution rates of $D = 0.15 \text{ h}^{-1}$, the cells enlarged, however the ratio of surface area to cell volume decreased with the stalk length regaining its characteristic dimensions (Fig. 3.33) (see Section 3.III.1).

Comparing the population of batch cultures with continuous cultures, it would appear that the low dilution rates, in part, mimic the stationary phase, there being a predominance of swarmer cells with few stalked and budding cells. This would suggest that under these conditions, the cell population is 'energy' limited, the consequences of which are that swarm cell development is repressed whereas swarmer cell production continues although at a much reduced rate. As the dilution rate is increased or the cells are inoculated into fresh medium, the increase in available nutrients restores the overall 'energy' levels to the point where cell development can resume, the swarmer cells developing stalks and ultimately buds.

In order to compensate for low growth rates, the cells respond by increasing the surface area to volume ^{presumably} ratio, to increase nutrient uptake. However, below a certain threshold 'energy' level swarm cell development, but not production, is repressed. Further studies are needed to confirm this model, to include higher dilution rates and consequently faster growth rates until the culture ultimately washes out. Studies with Caulobacter and Rhodomicrobium (Dow, personal communication) appear to give similar results of swarmer cell development being repressed by reduced 'energy' levels when studied in batch culture.

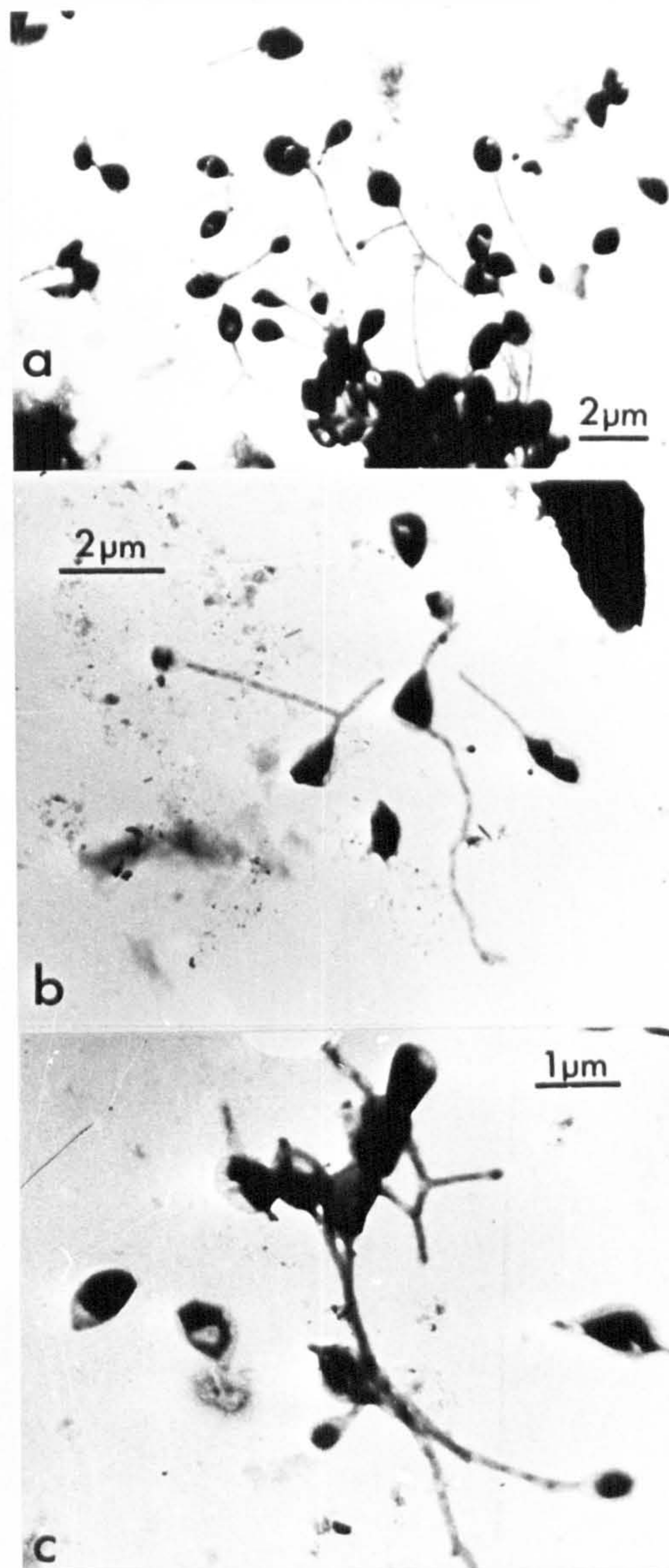


Fig. 3.32(a) Hyphomicrobium cell types observed in continuous culture at low dilution rates ($D = 0.02 \text{ h}^{-1}$). Cells are elongated, and stalked cells possess long stalks (see Text).

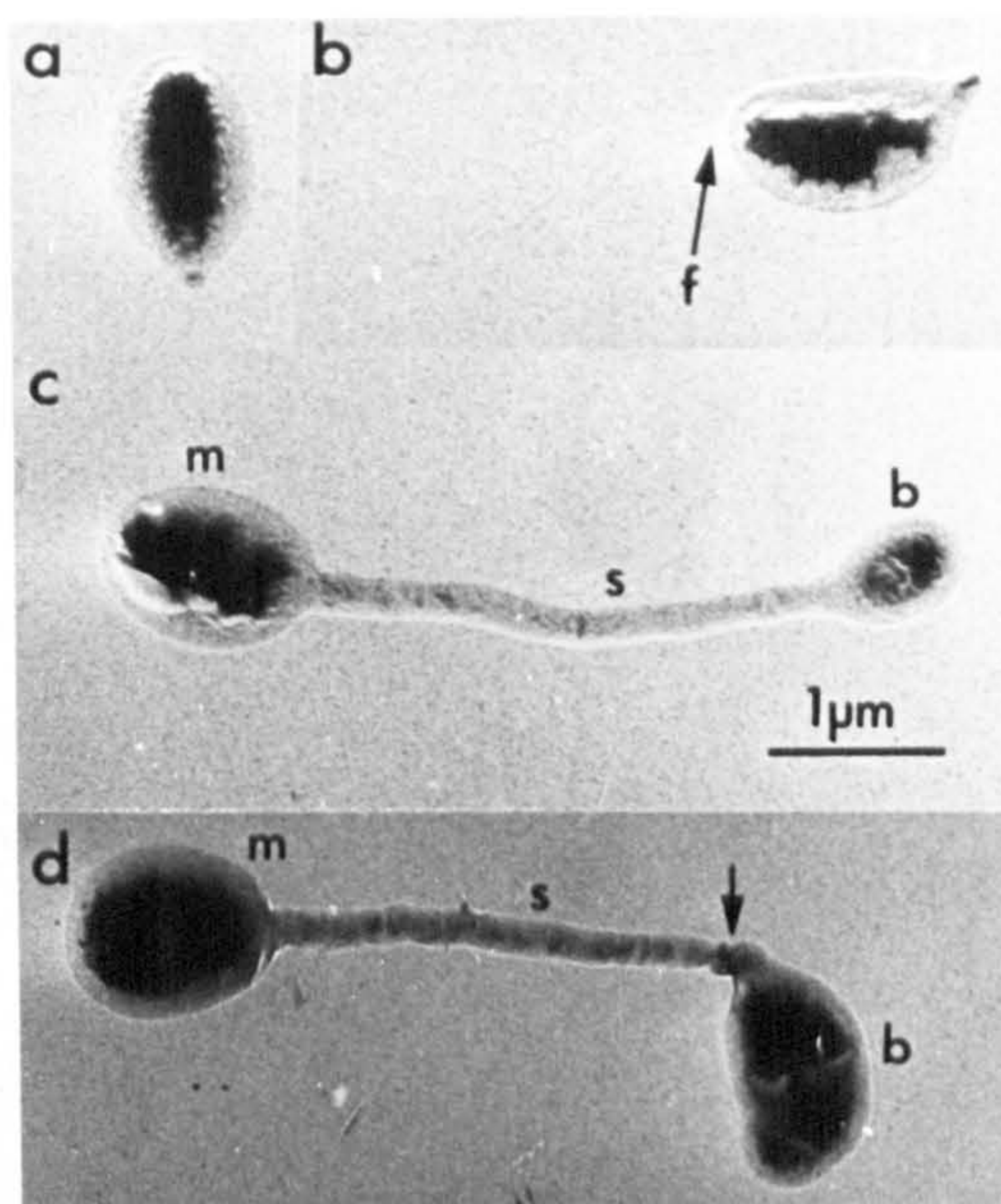


Fig. 3.32(b) Individual cell types of *Hyphomicrobium* in continuous culture at $D = 0.02 \text{ h}^{-1}$. (b) Note 'kidney-shaped' swarmer cell (arrowed) cf. normal (a).

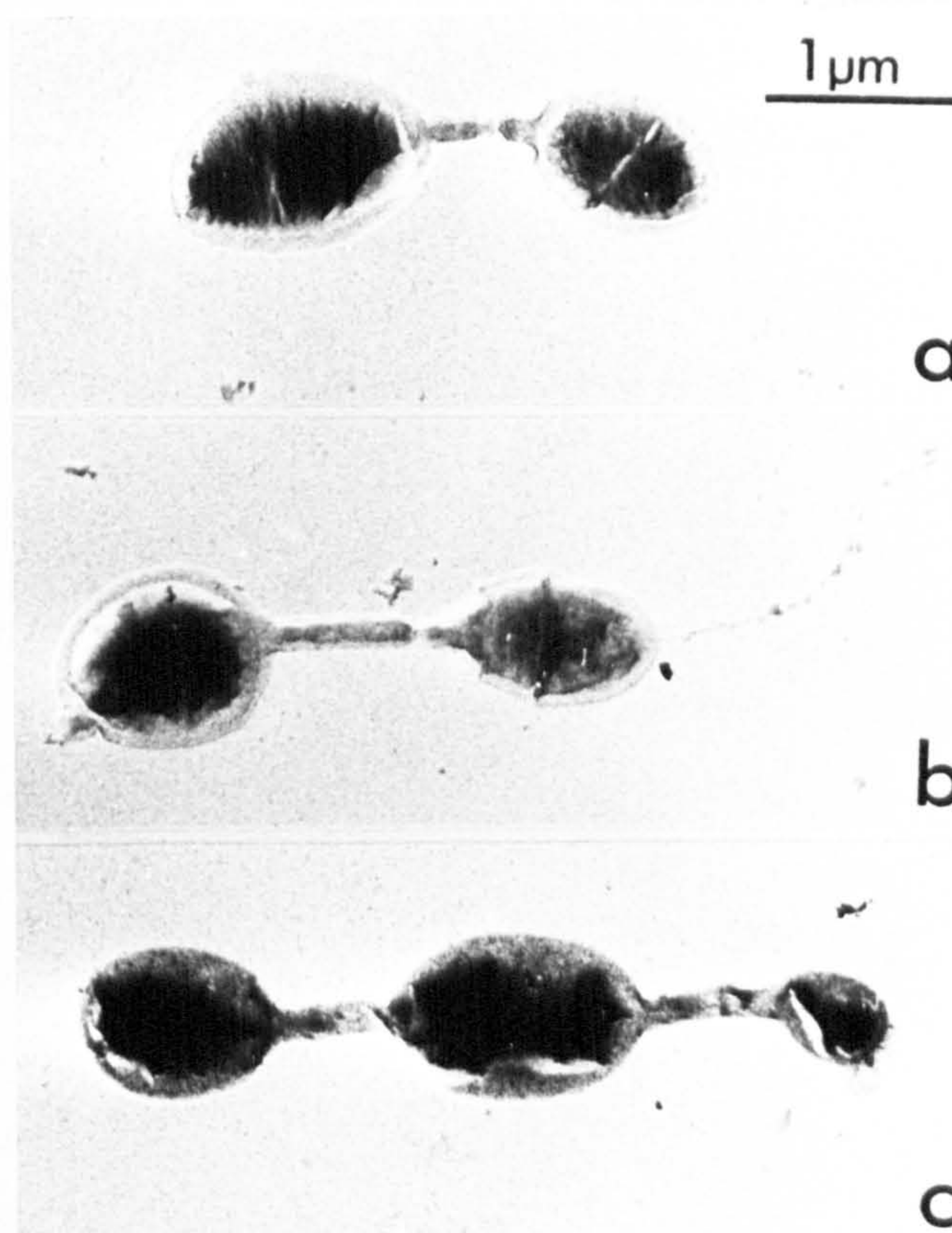


Fig. 3.33 *Hyphomicrobium* in continuous culture ($D = 0.15 \text{ h}^{-1}$). Cells are more rounded (a), and stalks appear shorter than at lower dilution rates, (b) and (c). Double stalked cells were not uncommon (c). Gold/Palladium shadowed.

Previous studies on bacterial population dynamics in continuous culture have mainly focussed on cells which have only a monomorphic growth cycle, e.g. E. coli (Koch and Coffman, 1970). The prosthecate bacteria, with their obligate dimorphic life cycles and environmentally induced pleomorphism, constitute more 'complicated' organisms, and consequently models of population kinetics must take into account the different cell types, and their individual response to growth parameters.

(d) Stalk synthesis

In order to determine the growth point of Hyphomicrobium cells, synchronous populations of cells were treated with penicillin and lysozyme mixtures, using a modification of the method of Schmidt and Stanier (1966). Spheroplasts were, however, difficult to stabilise despite the presence of polyethylene glycol, and the cells rapidly lysed. Microscopic analysis showed that the culture became sensitive to penicillin after 1 hour's incubation, and that spheroplasts developed at one pole of the swarmer cells and not randomly about the cell periphery, indicative of polar growth (Fig. 3.34a). When stalked cells were observed, spheroplasts only developed at the tip, never at the base or along the length of the stalk, indicating that stalk growth is from the tip of the stalk, throughout stalk synthesis (Fig. 3.34b and c). These results conflict with studies by Hirsch (1974) which indicate that initially growth is from the base of the stalk, and only later is stalk growth from the tip.

(e) Ultrastructure

The only ultrastructural studies on Hyphomicrobium that have been documented were by Zavarzin (1960) on Hyphomicrobium and by Conti and Hirsch (1965) on the fine structure of Rhodomicrobium and Hyphomicrobium spp. They showed that the majority of strains of hyphomicrobia possess a well-developed internal membrane system, which

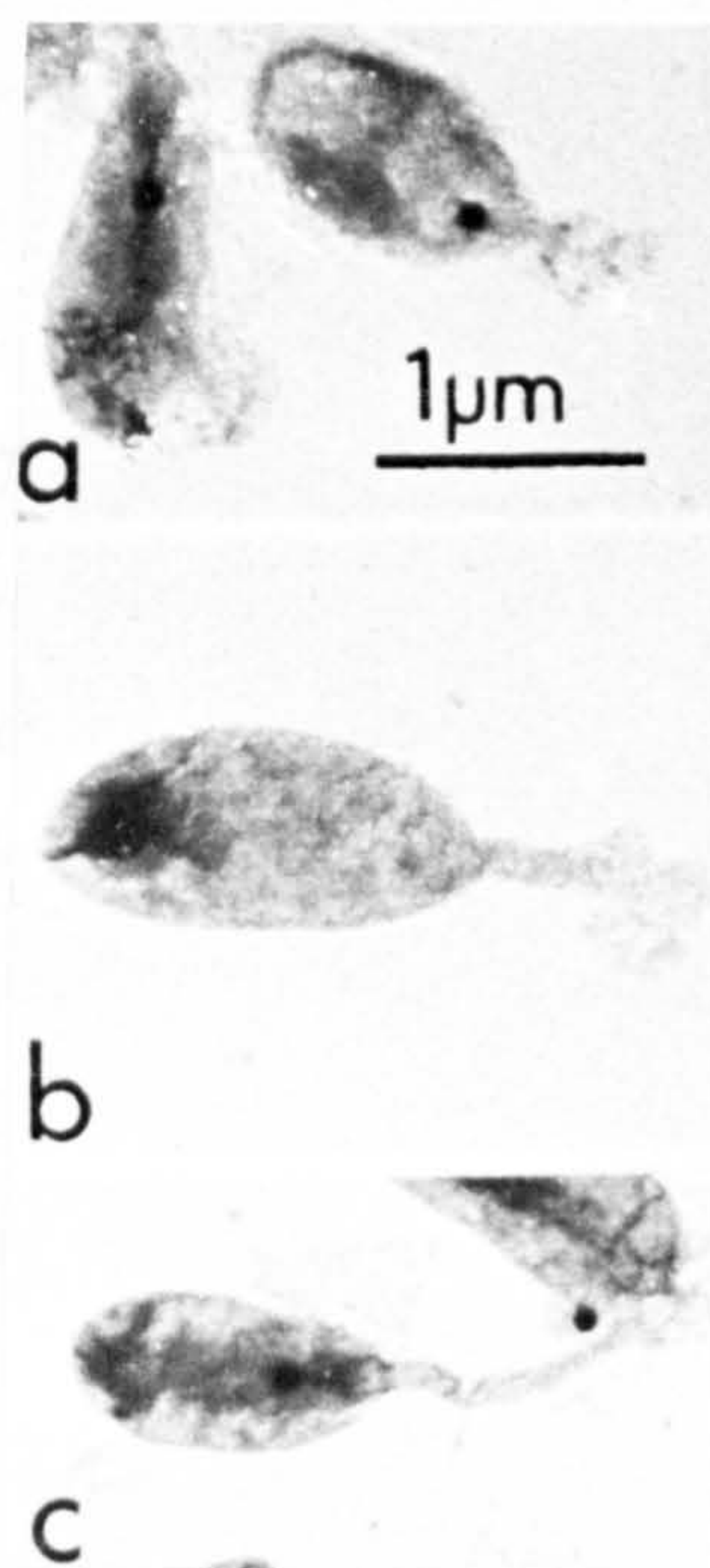


Fig. 3.34 Sphaeroplast formation in Hyphomicrobium. Sphaeroplasts formed at the site of cell synthesis, which appeared to occur at the polar region (a), and subsequently at the tip of the stalk (b) and (c).

appears to be derived by invagination of the cytoplasmic membrane. The type strain, H. vulgare, however, and a strain obtained by Zavarzin did not appear to have this intracellular membrane system.

Observations in this study on the fine structure of Hyphomicrobium are in agreement with those made on H. vulgare, that is the lack of such an intracellular membrane system (Fig. 3.35). As in many bacteria, the fibrillar nucleoplasmic regions and ribosomal particles constituted the principle internal elements of the cell (Poindexter and Cohen-Bazire, 1964, 1966); poly β -hydroxybutyrate granules were also present in some cells (Figs. 3.35, 3.36). The cell wall, cytoplasmic membrane and cytoplasm of the cell and stalk were continuous (Hirsch and Jones, 1968). In contrast to Rm. vanniellii (Dow et al., 1976), cross walls were not observed within the stalks. Although some strains of hyphomicrobia had been observed in rosette formation in liquid cultures, no holdfast material was detected in these ultrathin sections.

In considering the lack of a developed intracellular membrane system as described by Conti and Hirsch (1965), one must appreciate that it might not be absent, but poorly developed in the isolates sectioned in this study, under the conditions of growth employed. Alternatively, it may not be readily visible because of lack of contrast or inadequate specimen preparation, although this seems improbable as several isolates were prepared individually and none revealed an intracellular membrane system.

Ultrathin sections of Rm. vanniellii, grown under various conditions, showed that the lamellar membrane system may be present in varying degrees, i.e. membrane content varied inversely with light intensity, and that 'compartmentalisation' was frequently apparent (Whittenbury and Dow, 1977). This has been interpreted as being a reflection of the replication process (Whittenbury and Dow, 1977). Hyphomicrobium, in all stages of development, consistently showed 'compartmentalisation', agreeing with observations made by Zavarzin (1960), which possibly reflects the organised mode of growth and reproduction in this organism.

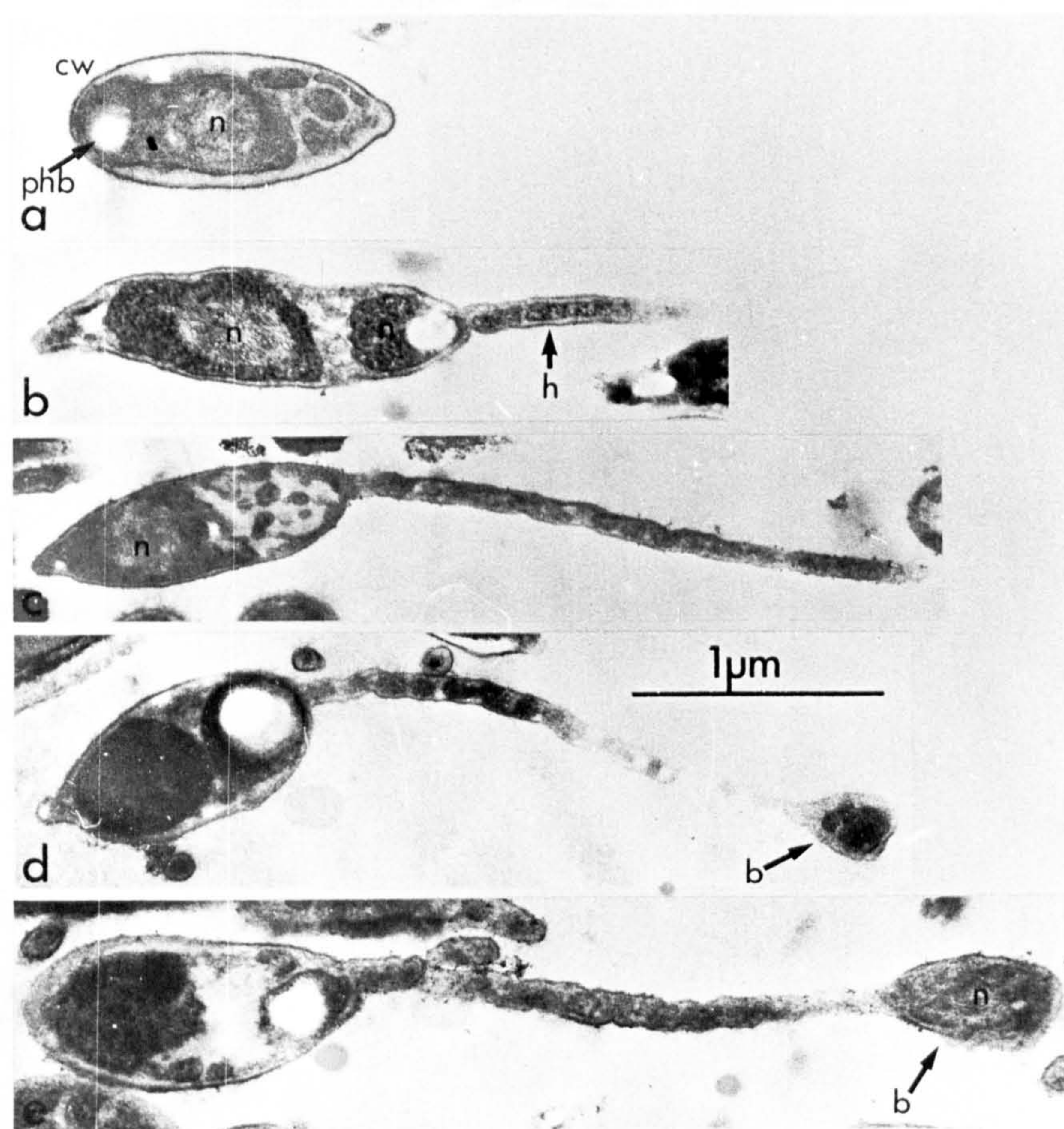


Fig. 3.35 Ultrathin sections of *Hyphomicrobium*, longitudinal sections, (a) swarmer cell, illustrating internal compartmentalisation of the internal components, (b) stalked cell with two functional units, (c) daughter cell genome is drawn up the stalk as it grows out, (d) mother cell and bud, (e) as bud develops, genome in mother cell starts to replicate again.

(h = hypha or prostheca, n = nuclear material, b = bud, phb = poly β -hydroxybutyrate, cw = cell wall).

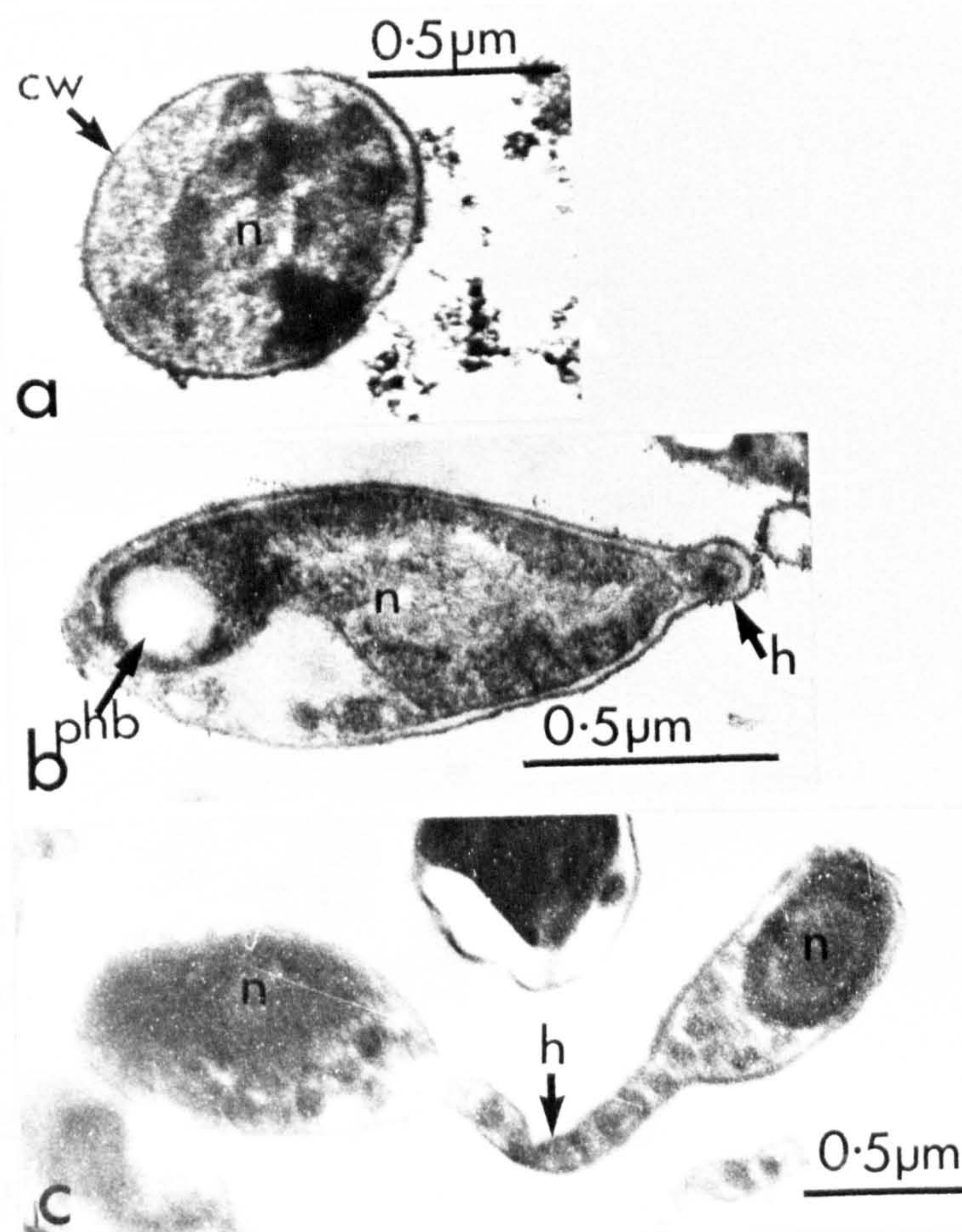


Fig. 3.36 Ultrathin sections of *Hyphomicrobium*. (a) transverse section shows lack of any intracellular membrane system. (b) mature swarmer cell with fibrillar nuclear material and poly β-hydroxybutyrate. (c) mother cell with mature bud, before division. (n = nuclear material, phb = poly β-hydroxybutyrate, cw = cell wall).

(Fig. 3.35). The compartmentalisation of the mother cell can be explained as cellular replication and division within the confines of the cell wall. The daughter cell is preformed within the mother cell and enclosed in its own cytoplasmic membrane, and extends into the stalk as a functional unit, comprising of DNA, cytoplasm and cytoplasmic membrane (Fig. 3.35 b). On completion of the first daughter cell 'unit', the mother cell commences a second reproductive process within the confines of its own cell volume (Fig. 3.35 e). The genome appears to be actively transported into the new cell by the cytoplasmic membrane, rather than by streaming of the cytoplasm as has been suggested (Moore and Hirsch, 1973). Thus reproduction in Hyphomicrobium appears to resemble the model proposed by Whittenbury and Dow (1977) for Rhodomicrobium, where each daughter cell formed is dependent on a period of filament synthesis, in order to divide the DNA attachment point (Section 2.1).

III Bacteriophage for Hyphomicrobium

Despite numerous attempts to concentrate down water samples from a variety of environments, using methods described for prosthecates (Stanley et al., 1976), no 'phage could be found for Hyphomicrobium species tested.

To date only one 'phage has been isolated for Hyphomicrobium, designated Hy Ø30 (Gerenscer and Voeltz, 1971). This was a double-stranded DNA 'phage resembling Salmonella 'phage P22. It appeared to adsorb only to the tip of the growing stalk and to the developing daughter cell. The motile cell was immune to 'phage infection (Voeltz, Gerenscer and Kaplin, 1971). These authors suggested that a correlation exists between synthetically active cytoplasmic areas, receptor sites and 'phage replication, the latter only being seen in daughter cells and stalks, not in mother cells.

Despite the ubiquitous nature of Hyphomicrobium in the natural environment (Hirsch, 1974), no other 'phage have been isolated to date, although new concentration methods are currently being employed in these laboratories to try and overcome the elusiveness of this 'phage.

IV Pleomorphism in *Hyphomicrobium*

Studies on *Hyphomicrobium* in the natural environment and in pure culture have shown that this bacterium is pleomorphic (Hirsch and Conti, 1964; Tyler and Marshall, 1967b; Bauld, Tyler and Marshall, 1971). The following studies were undertaken in order to ascertain whether the pleomorphic forms observed in *Hyphomicrobium* cultures were artefacts or should be included in the description of the genus (cf. Hirsch, 1974). The effects of carbon and nitrogen source variations, together with certain heavy metals, on cellular expression in this prosthecate bacterium were considered and the results related to previous observations (Tyler and Marshall, 1967; Hirsch, 1968; Tyler, 1970).

(a) Carbon variations and 'lobed' cells

The ability of *Hyphomicrobium* to grow on methanol and methylamine has already been demonstrated (Section 3. II. 1). Substituting one carbon source for another did not appear to affect cell growth significantly, however it did give rise to morphological variations. With methanol as the carbon source, the majority of the cells were classically pear-shaped with buds at the ends of long, slender stalks (Fig. 3.2). Occasionally in the late exponential or stationary phase of growth, the culture included lobed cells (Fig. 3.38) which were normally observed free, although occasionally attached to mother cells (Fig. 3.37). When methylamine, partly or completely, replaced methanol as the carbon source, the cells became very pleomorphic. Not only did the number of trilobed cells increase, but some produced bizarre cell forms by undergoing dichotomous branching (Fig. 3.38). Other cells became elongated especially in mixed carbon medium (methanol and methylamine) and contained large granules of poly β -hydroxybutyrate (PHB), (Bauld, Tyler and Marshall, 1971). These cells, as well as displaying cellular pleomorphism, also exhibited colonial pleomorphism. The stalks became branched, stalks forming from several locations on the cell body, and sessile budding was also observed (Fig. 3.39). In methylamine supplemented liquid cultures, chains and multicellular arrays of cells were formed, in agreement with observations made by Bauld, Tyler and Marshall (1971). After sustained subculturing

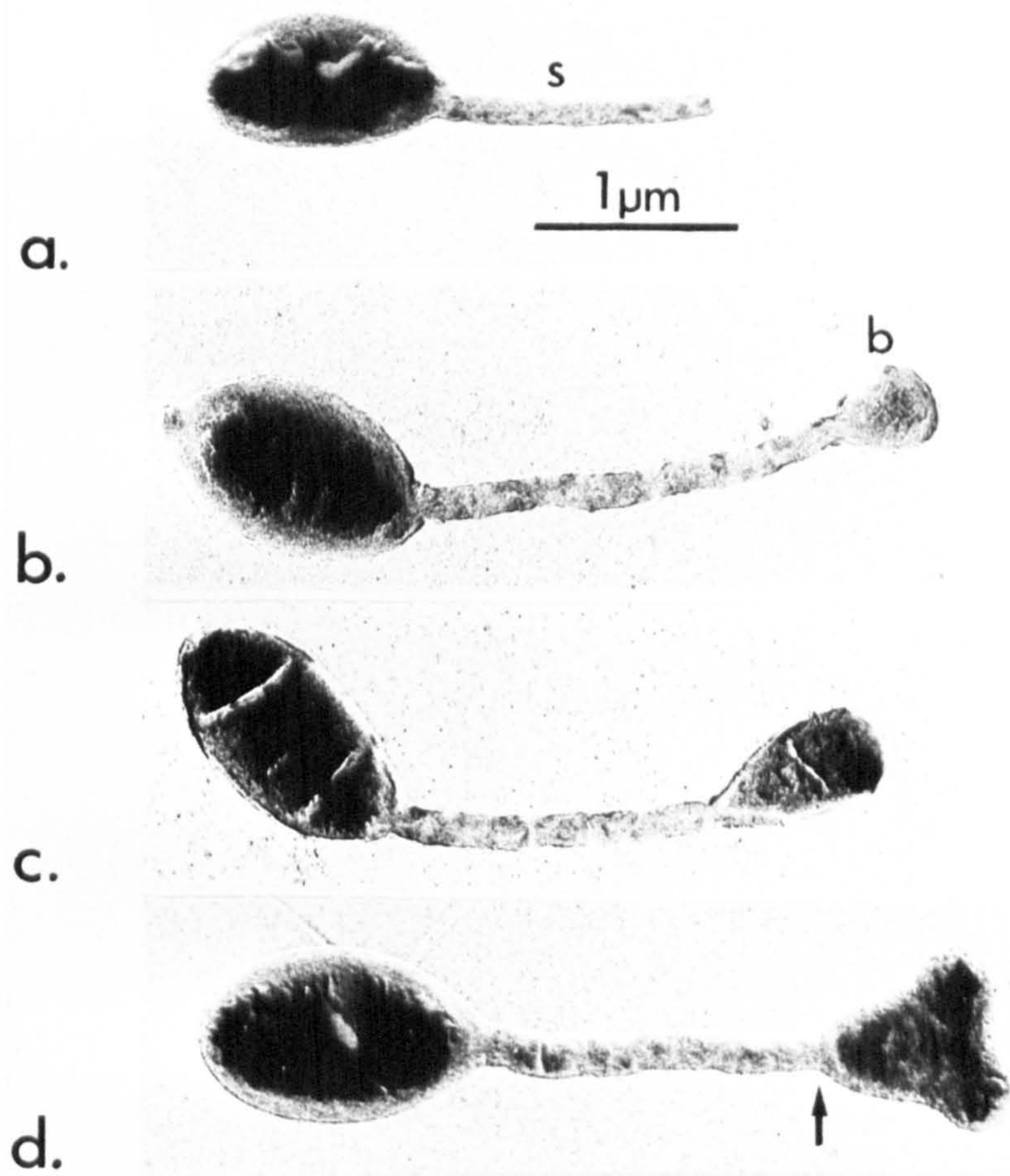


Fig. 3.37 Development of lobed cells from 'normal' cells. Arrow indicates plane of division. (Gold/Palladium shadowed).

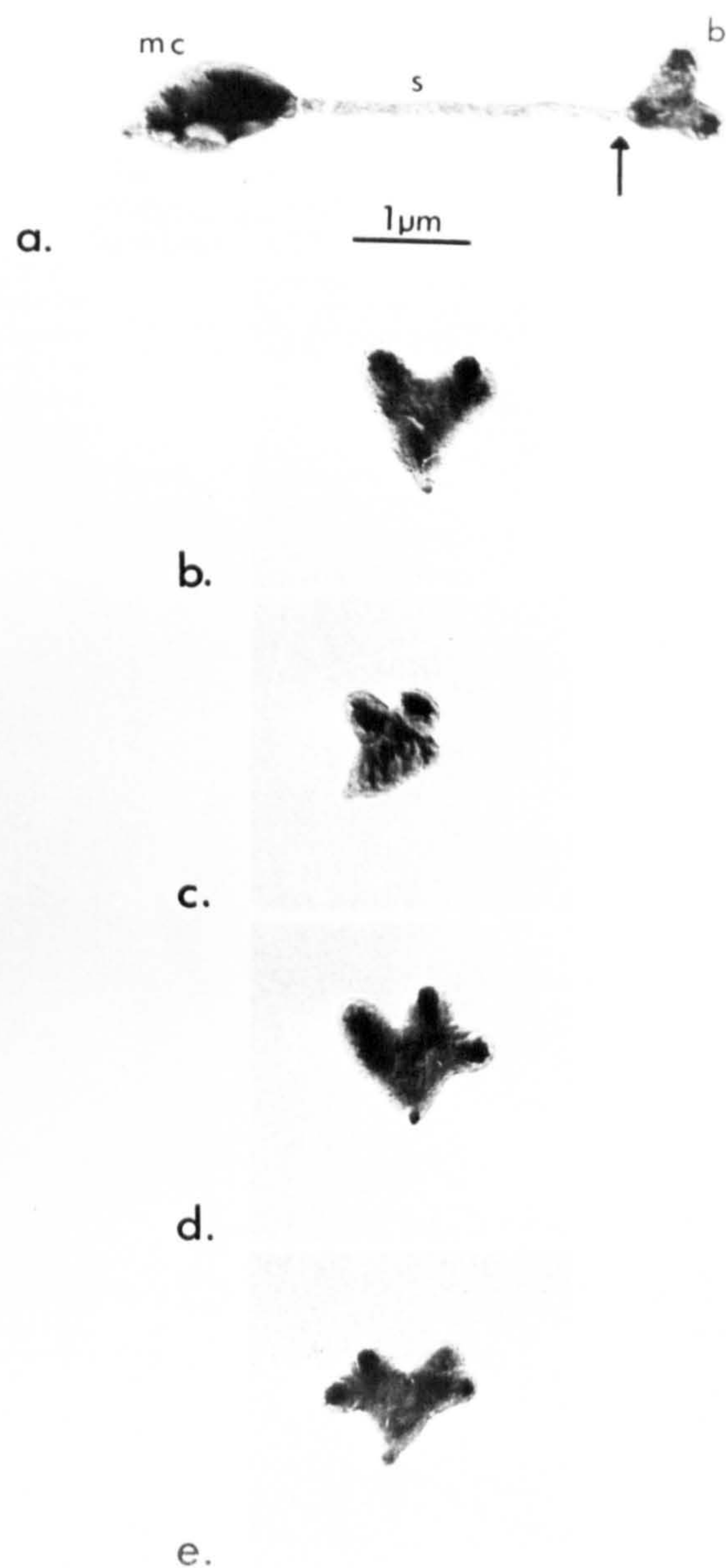


Fig. 3.38 Dichotomous lobing of the lobed cell, giving rise to trilobed cells (d). (Gold/Palladium shadowed).
(mc = mother cell, s = stalk or prostheca, b = bud, arrow = plane of division).

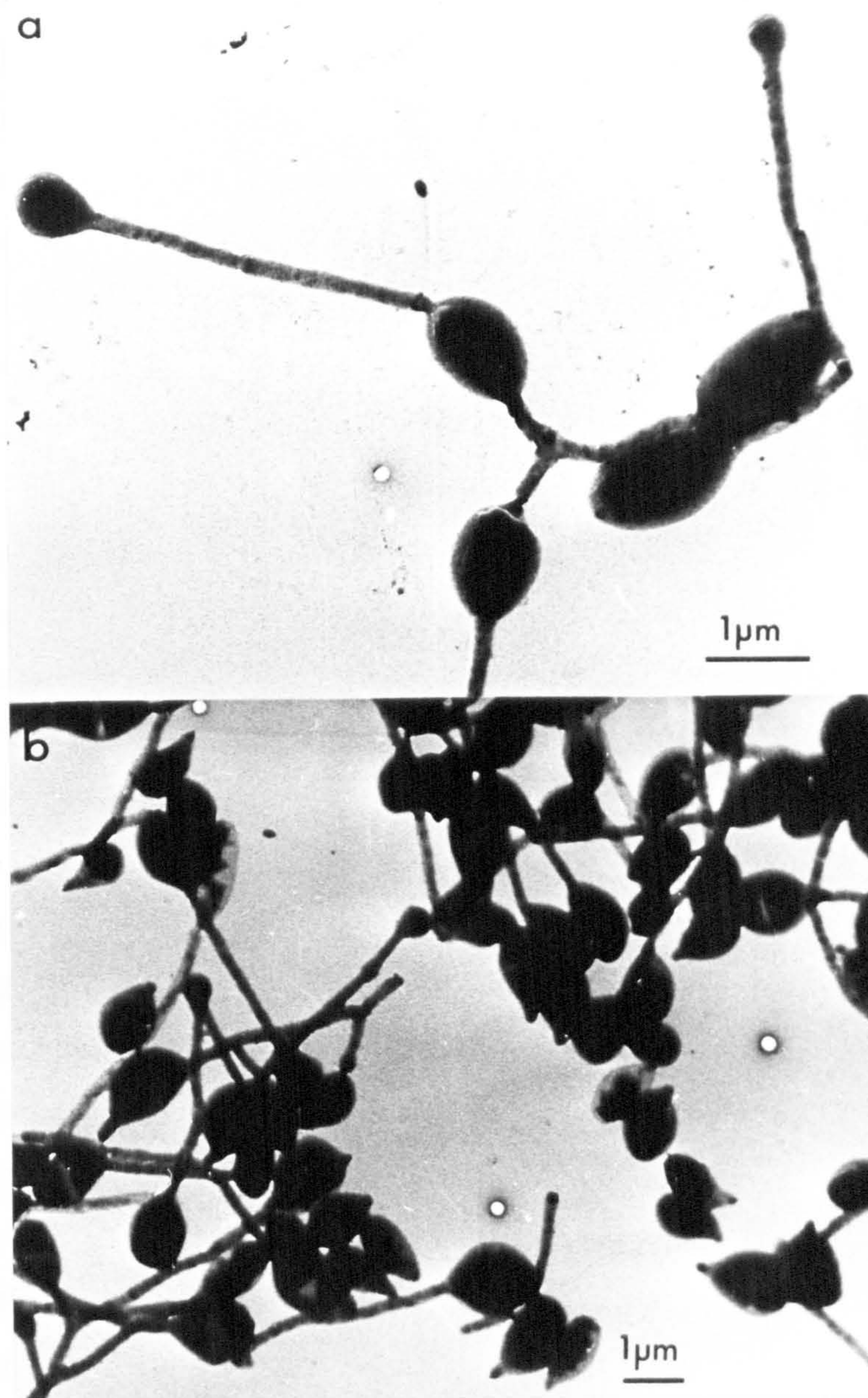


Fig. 3.39 Colonial pleomorphism in Hyphomicrobium cultures growing in mixed carbon medium (methanol and methylamine). Stalks become elongated and branched, and buds, when formed, remain attached, forming multicellular arrays.

with methylamine as sole carbon source, the cells returned to their classical morphology, however with methanol and methylamine together, trilobed cells as well as lobed cells could be maintained in the culture for some time and were never completely lost.

To determine whether there was any significance in the lobed cells being maintained in the presence of methanol and methylamine, the incorporation of ^{14}C labelled CH_3OH and $\text{CH}_3\text{NH}_2\cdot\text{HCl}$ was monitored over a complete batch growth cycle. Growth of Hyphomicrobium on either carbon substrate gave comparable results, however a mixture of both carbon compounds initially gave a rate of label incorporation that followed the rate for $\text{CH}_3\text{NH}_2\cdot\text{HCl}$ and then continued to give a rate of incorporation similar to that for CH_3OH when used as sole carbon substrate (Fig. 3.40). To ascertain as to whether this suggested diauxic growth, mid-exponential cells grown in 0.5% (v/v) CH_3OH were inoculated into medium containing 100 $\mu\text{Ci/ml}$ $^{14}\text{CH}_3\text{NH}_2\cdot\text{HCl}$ and 0.1% (v/v) 'cold' CH_3OH , and rates of incorporation of label were monitored. Similarly cells grown in 0.5% (v/v) $\text{CH}_3\text{NH}_2\cdot\text{HCl}$ were inoculated into medium containing 100 $\mu\text{Ci/ml}$ $^{14}\text{CH}_3\text{OH}$ and 0.1% (v/v) 'cold' $\text{CH}_3\text{NH}_2\cdot\text{HCl}$, and monitored for the incorporation of label (Fig. 3.41). This study demonstrated that with the mixed substrate, $\text{CH}_3\text{NH}_2\cdot\text{HCl}$ was used preferentially to CH_3OH , however CH_3OH appeared to give optimal growth in batch culture (Fig. 3.6). Possibly this diauxic effect directly or indirectly effects the morphological changes observed in cultures with methylamine and methanol as carbon substrates, by altering certain parameters within the cell, as has been described in studies on Geodermatophilus (Ishiguro and Wolfe, 1970).

Cellular pleomorphism - lobed cells

Like the classical cell type, lobed Hyphomicrobium cells possess an obligate differentiation cycle. Once released from a classical mother cell (Fig. 3.42), this pleomorphic cell can develop in several ways. Firstly, the lobed cell can undergo repeated dichotomous

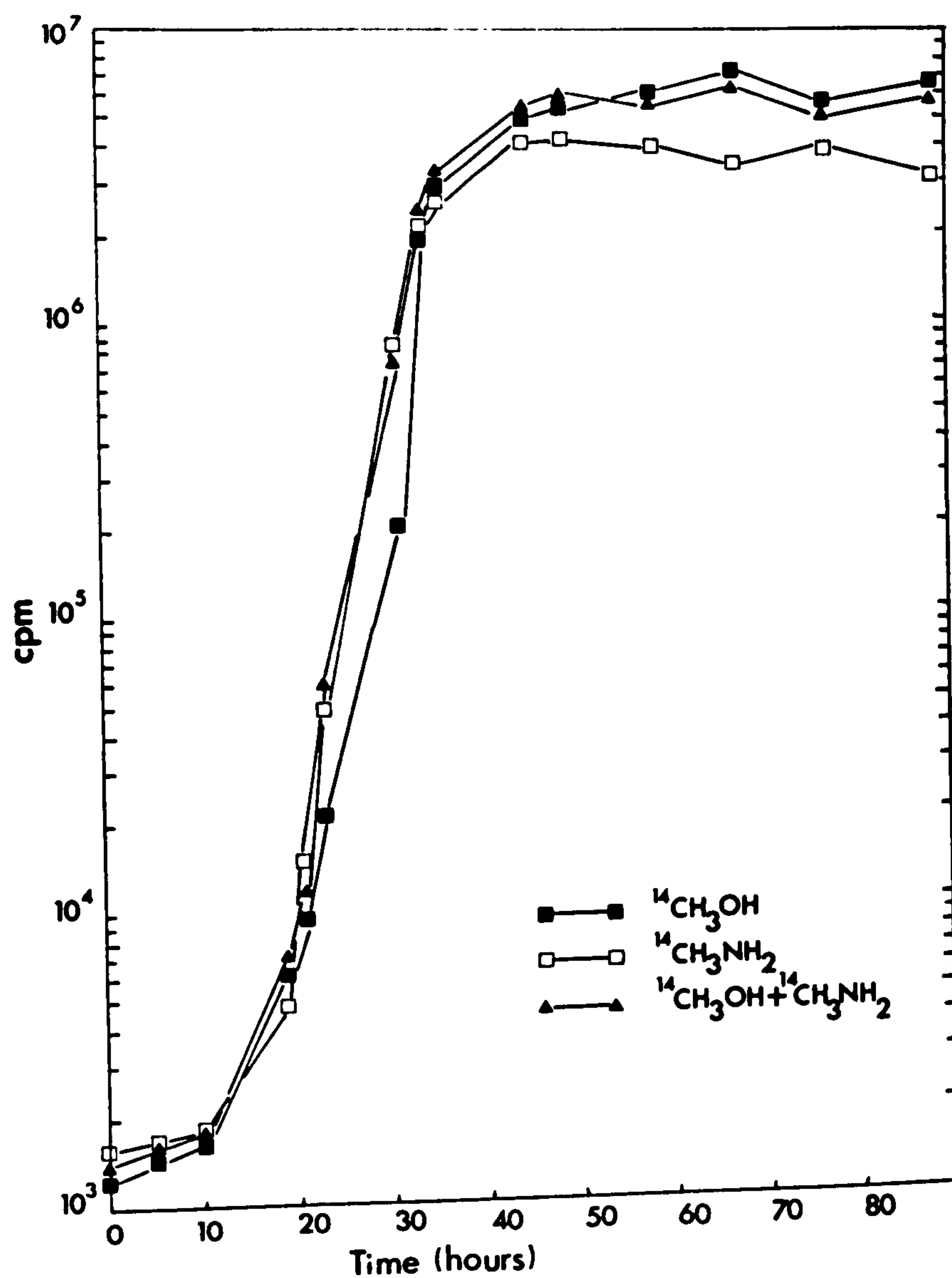


Fig. 3.40 Uptake of labelled carbon substrates by Hyphomicrobium .
A mixture of methanol and methylamine gave incorporation rates of each carbon source, suggesting possible diauxic growth.

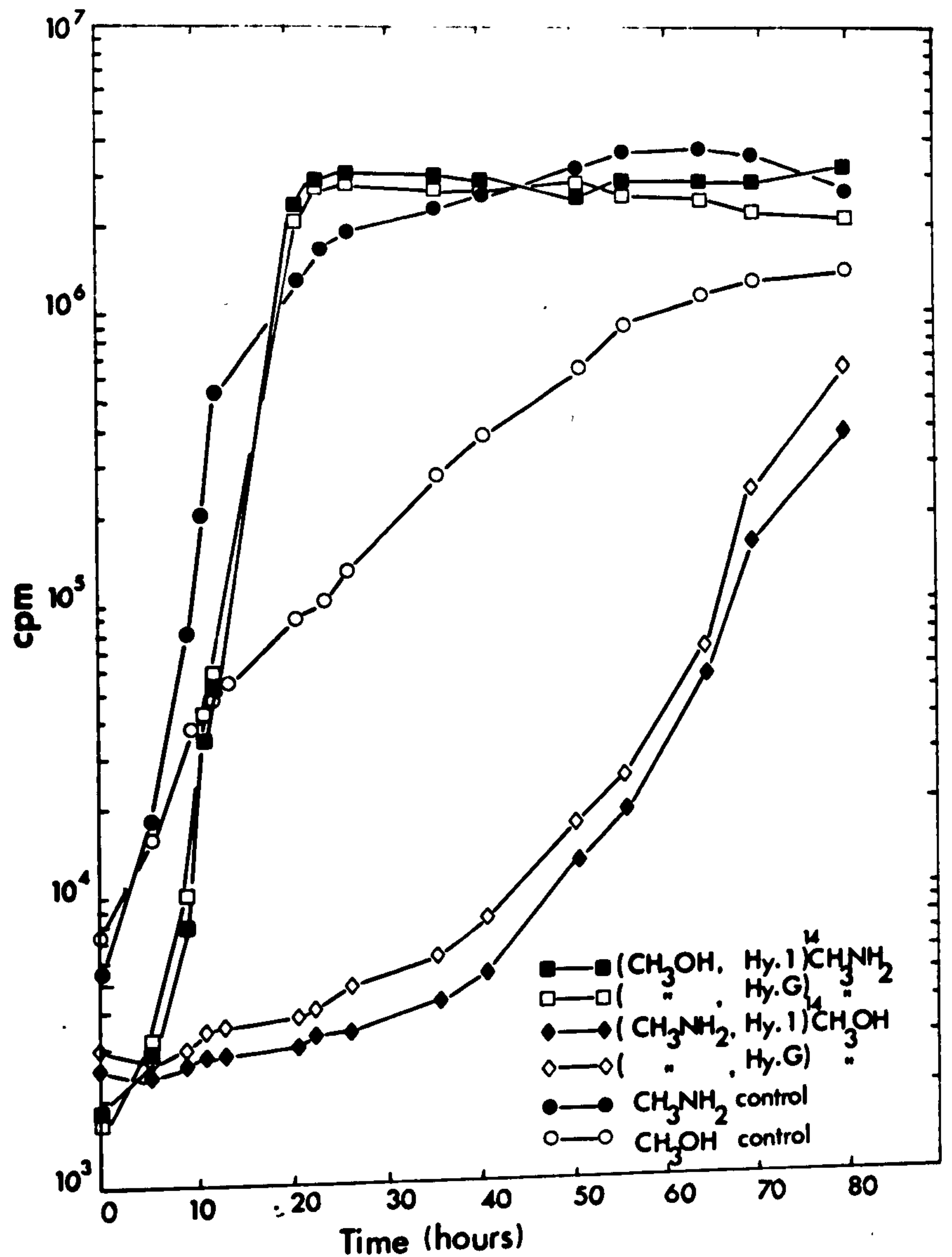


Fig. 3.41 Using isolates 1 and G of *Hyphomicrobium*, incorporation of ^{14}C labelled CH₃OH in the presence of 'cold' CH₃NH₂, and ^{14}C labelled CH₃NH₂ in the presence of 'cold' CH₃OH were monitored. Controls were incorporation of label with no cold carbon substrate present. Methylamine appears to be used preferentially. With ^{14}C CH₃OH the slow rate of incorporation is due to the competitive uptake of 'cold' CH₃NH₂

lobing (Figs. 3.38, 3.43). Normally one of the lobes of the cell will divide to form two smaller lobes, which gradually increase in size, then the other lobe starts to divide in a similar manner. Only one lobe can develop at a time, analogous to only one stalk developing a bud in the classical cell cycle, when the mother cell is bistalked. When the dividing lobes attained a critical size, they appeared to be pinched off from the mother cell, so becoming independent lobed cells. Secondly, the lobed cell can produce a stalk, similar to that found in the classical life cycle, from one of its lobes. From this stalk can develop further lobed cells (Figs. 3.44, 3.46) or classical buds (Figs. 3.45, 3.46). These lobed cells appeared physiologically identical to the 'normal' cells and did not appear to possess any properties attributed to resting cells, e.g. resistance to heat or dessication.

This example of cellular pleomorphism differs from the morphologically different forms found within the 'normal' life cycle of Hyphomicrobium (Fig. 3.20) in that these lobed cells can be environmentally induced, that is by alteration of the nutrient status of the medium. The nutrient status alters during the growth cycle, as the medium becomes depleted, or alternatively it can be deliberately disturbed by altering the composition of the medium, i.e. by replacing methanol with methylamine. Cellular pleomorphism which can be induced or repressed by the environment contrasts sharply with the different morphological forms found in the 'normal' life cycle. The latter types are obligate expressions of the developmental cycle. Although the lobed cell is a pleomorphic form of Hyphomicrobium, not essential to its survival, it too must undergo obligate differentiation (Figs. 3.43, 3.46, 3.47).

A possible triggering device for the expression of these lobed cells could be the intracellular pH, which one would imagine to have a marked effect upon cellular mechanisms. Studies showed that the pH of methanol cultures remained fairly constant (pH 6.7 - 7.2), whilst with methylamine the pH would drop to pH 5.0 or lower by the time the culture was in stationary phase. If methylamine is taken up by the

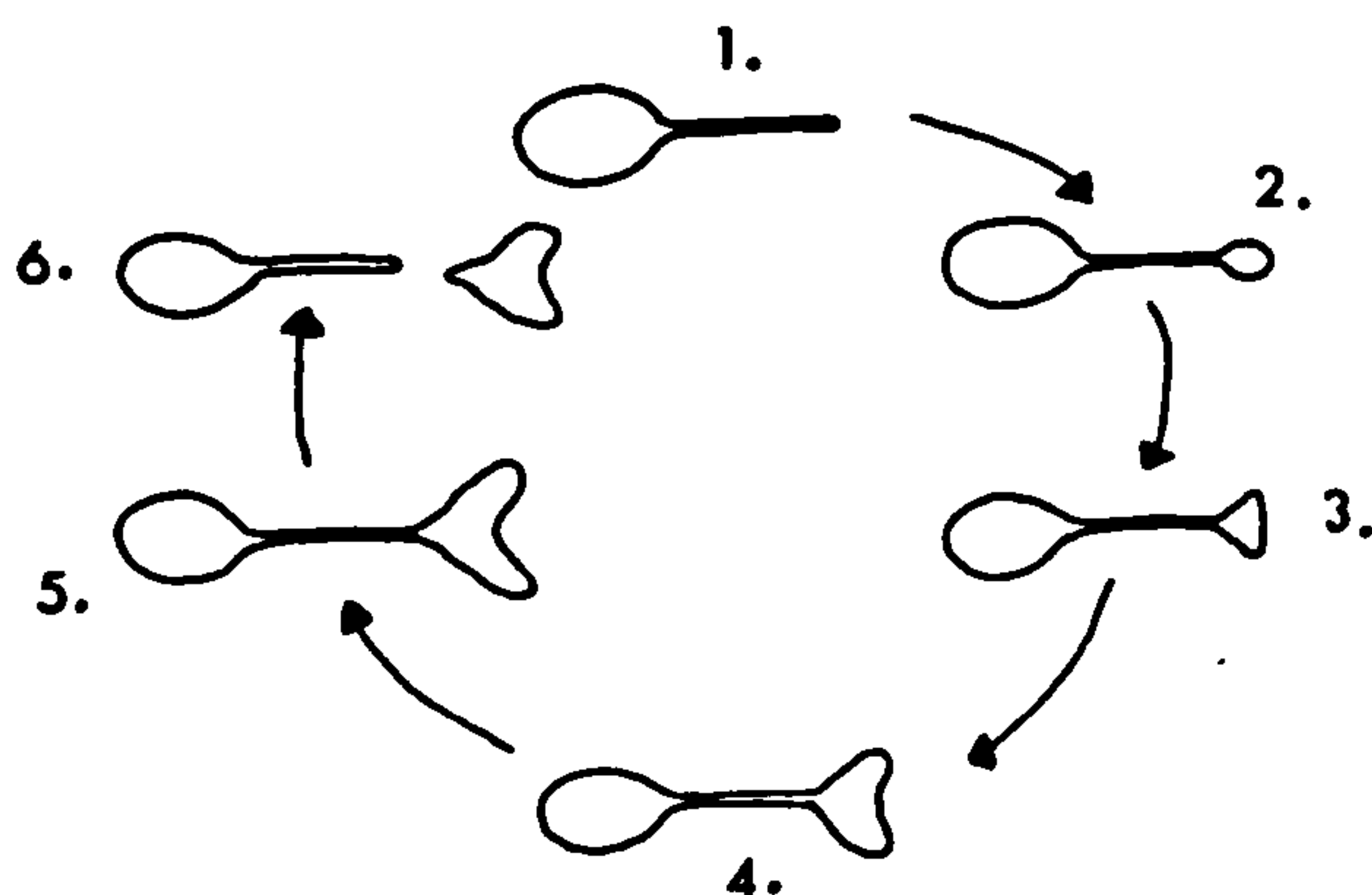


Fig. 3.42 Formation of lobed cells from 'normal' cells .

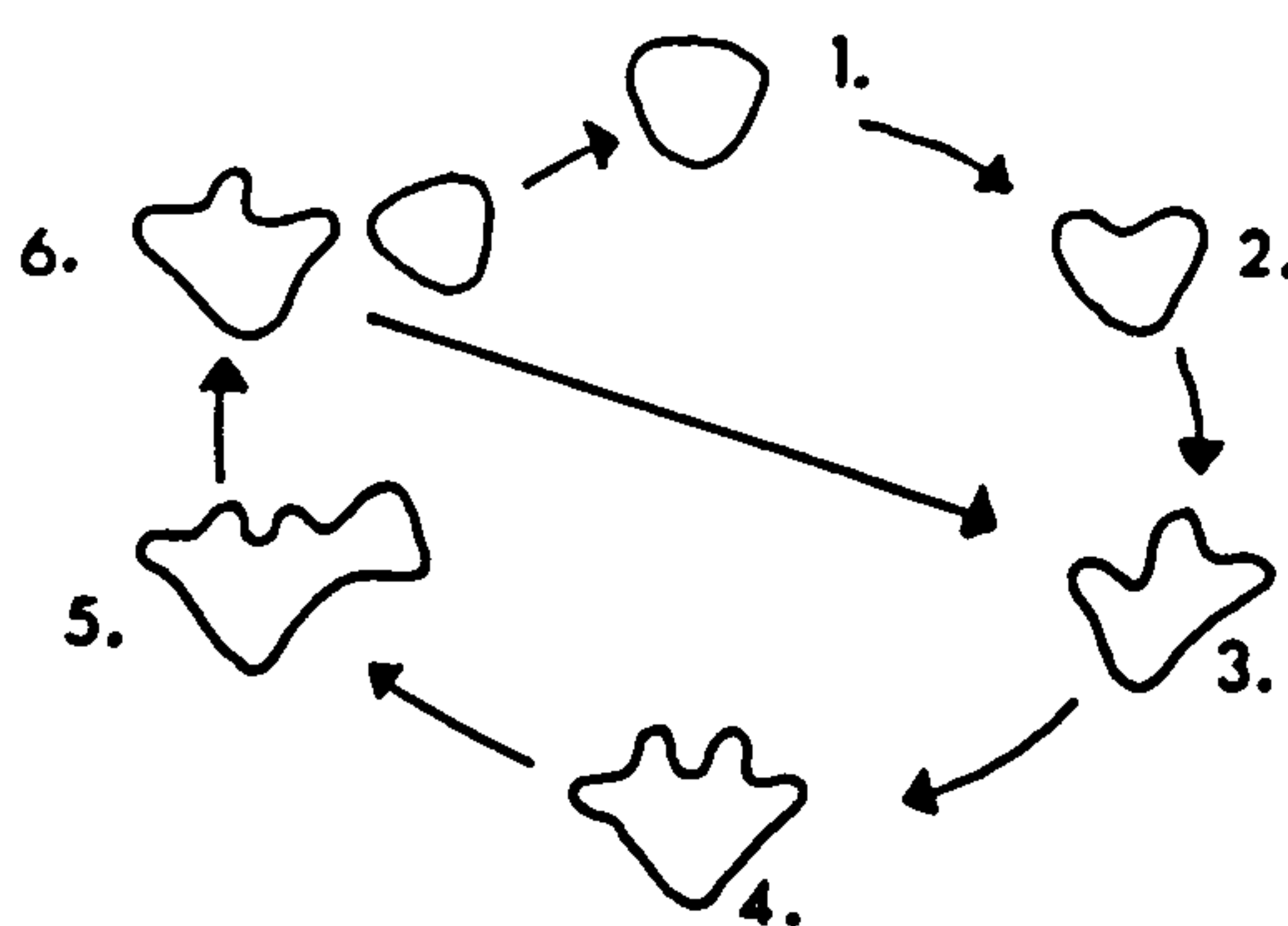


Fig. 3.43 Life cycle of lobed cells as a phenotypic variant of Hyphomicrobium.

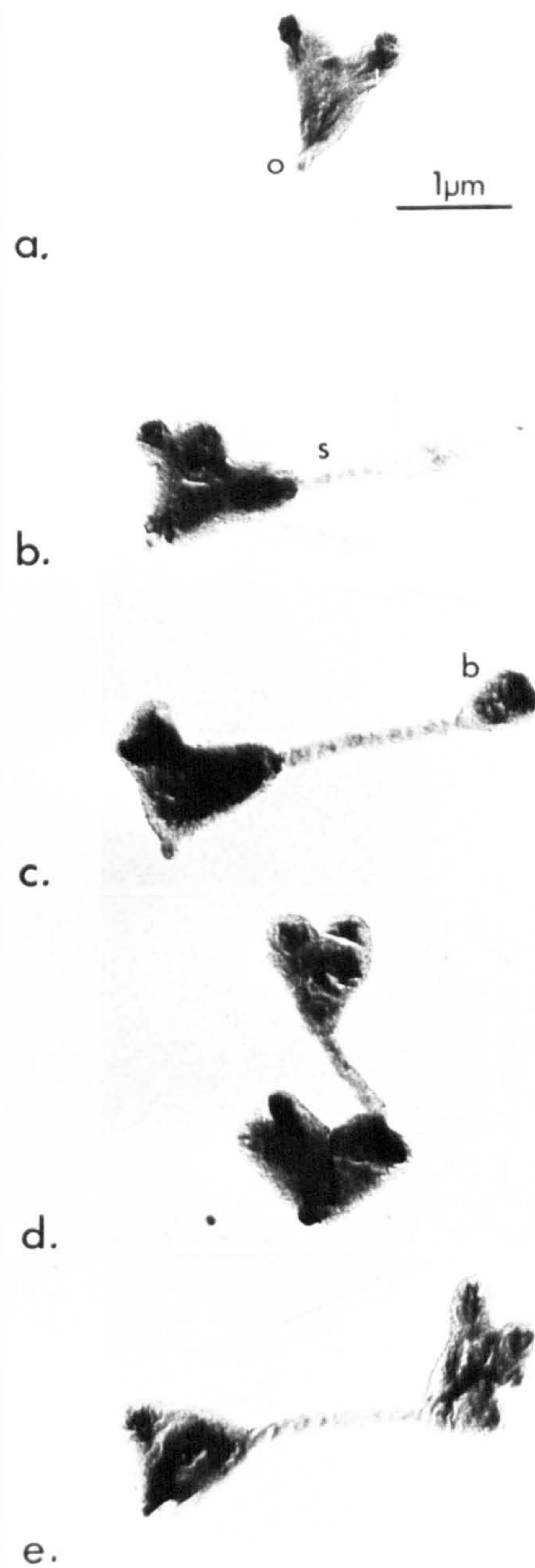


Fig. 3.44 Stalk formation in lobed cells, giving rise to lobed cell buds. [(o) represents the point of origin with the stalk of the mother cell, (s) is the stalk and (b) the bud]. In this case the lobed cell buds off further lobed cells.

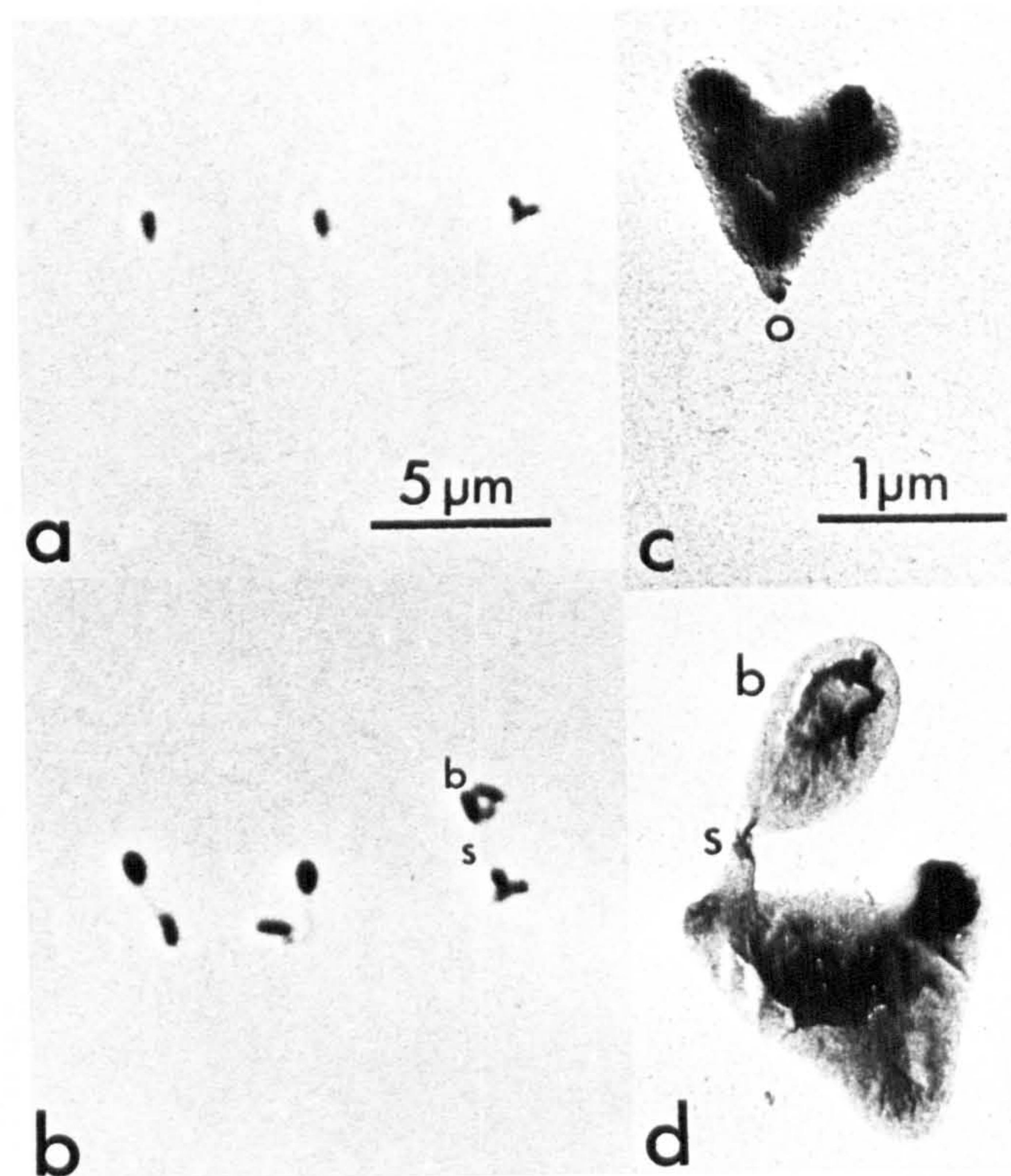


Fig. 3.45

Lobed cell producing 'normal' cells, from its cellular stalk (d). Slide culture illustrates that the lobed cells grew normally, giving rise to the three daughter cells after 24 hours growth (b) (b = bud, s = stalk)

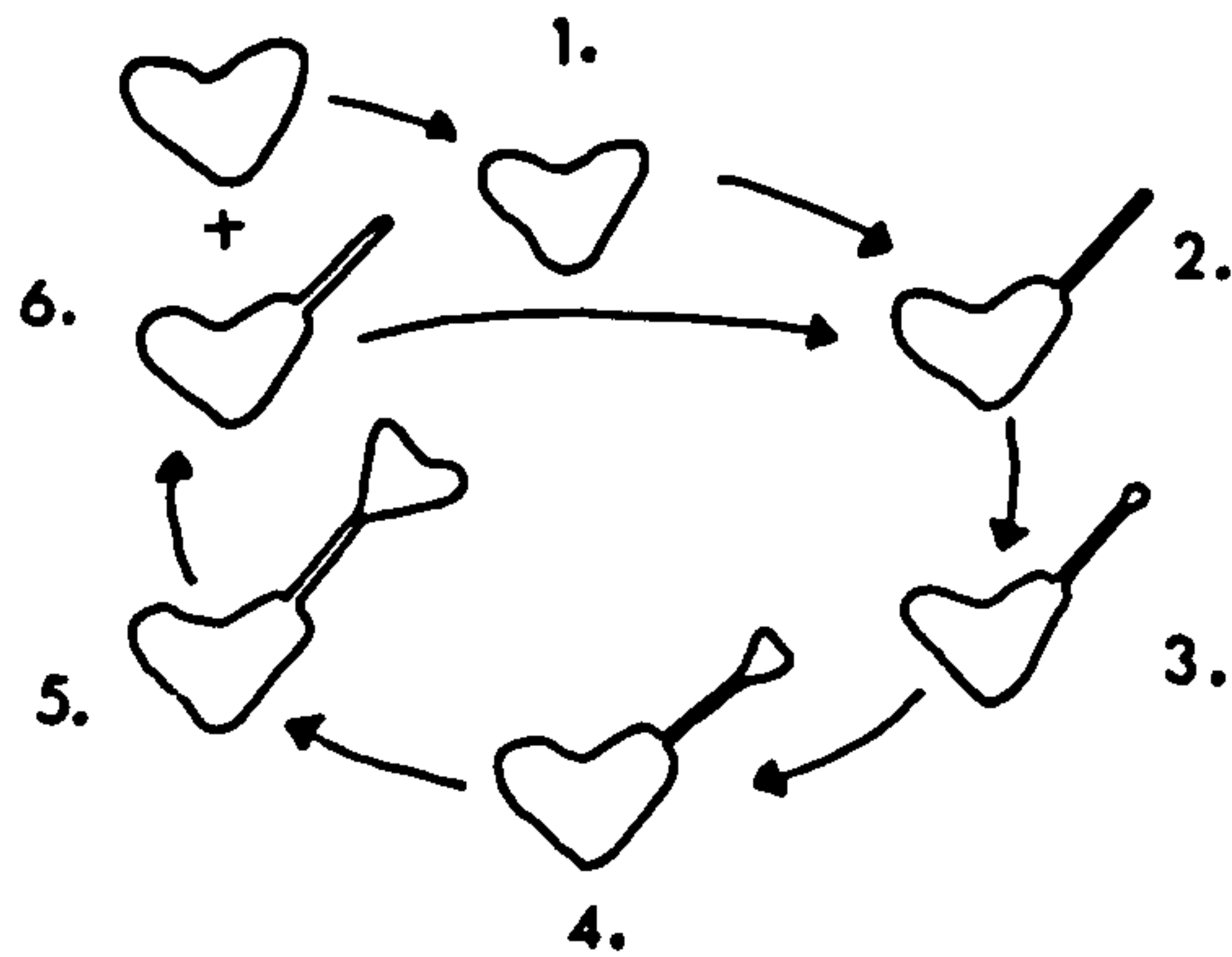


Fig. 3.46(a)

Dimorphic life cycle of lobed cells, producing further lobed cells as buds from their stalks.

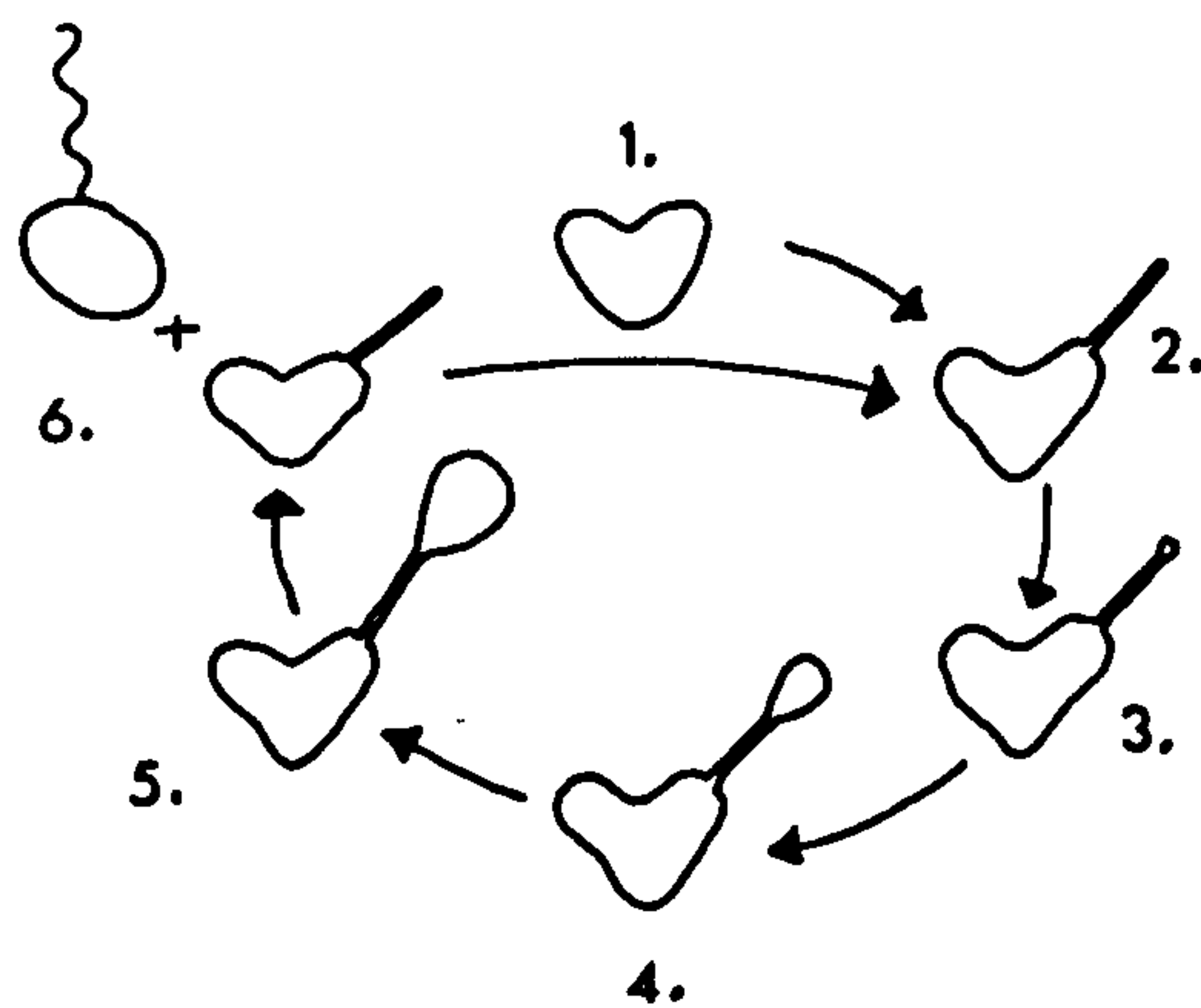


Fig. 3.46(b)

Life cycle of lobed cells 'reverting', to give rise to the normal cell type, the characteristic swarmer cell.

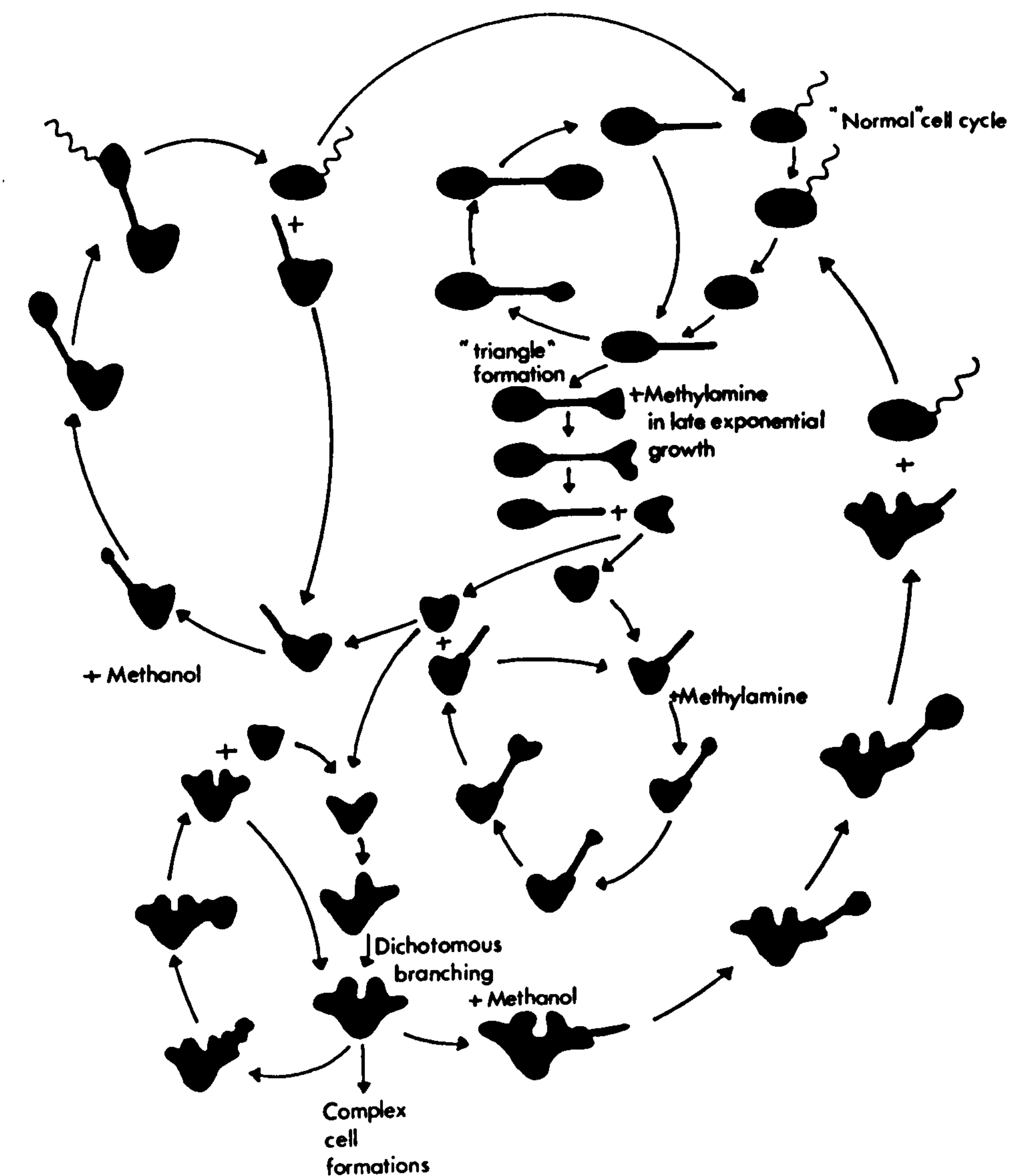


Fig. 3.47

Summary of the
Hyphomicrobium .

cellular pleomorphism in

cells, the methyl and amine moieties can be utilised for growth, consequently the cells excrete H^+ ions, causing the pH of the liquid culture to become acidic. Studies have been carried out in Geodermatophilus (Ishiguro and Wolfe, 1970, 1974) in which inorganic cations have been implicated in affecting the pleomorphism of this organism (Fig. 1.2). The level of cations employed was high (100 mM +), and although they caused retardation of growth, at these concentrations they were highly efficient inducers. With Geodermatophilus, the cations NH_4^+ , Na^+ and K^+ were efficient in maintaining the C form (Fig. 1.2). However, even though the inducing cations were taken up by differentiating cells, induction was not said to be solely dependent on high intracellular cation concentrations.

Accumulations of extruded H^+ in exchange for cations is assumed to account for the decrease in culture pH in these Geodermatophilus studies, with NH_4^+ shown to cause the greatest acidification. Studies on Streptococcus faecalis (Zarlengo and Abrams, 1963) demonstrated that NH_4^+ was passively transported as the free base NH_3 , the dissociated H^+ remained in the external medium, causing a decrease in the pH. On the basis of this, organic amines with strong basic properties, capable of penetrating the cell membrane, should be taken up passively, in the form of the dissociated base, with the accumulation of dissociated H^+ lowering the pH of the culture (Zarlengo and Abrams, 1963). Although inorganic cations and organic amines were taken up and implicated in the induction of pleomorphism in Geodermatophilus, Ishiguro and Wolfe stated that NH_4^+ and organic amines were not metabolised as the nitrogen or carbon source, and could only be shown to cause an increase in the intracellular pH, upon their uptake.

Inorganic cations and organic amines were added to methanol, methylamine and methanol-methylamine supplemented cultures of Hyphomicrobium, at various concentrations (Table 3.3), in order to determine whether they would affect the pleomorphism of this bacterium. Apart from the organic amines, which are known to support the growth of Hyphomicrobium as carbon and nitrogen source (Harder et al., 1978), the

Table 3.3 Effect of inorganic cations and organic amines on the growth and morphology of *Hyphomicrobium*

<u>Compound</u>	<u>Concentration tested (mM.)</u>	<u>Growth</u>	<u>Morphology</u>
K ⁺	100 mM	fair	few lobed cells, 5%
Na ⁺	100 mM	fair	few lobed cells, 5%
NH ₄ ⁺	100 mM	good	few lobed cells, 5%
Fe ²⁺	1, 5, 10, 100 mM	poor, none at 100 mM	lobed cells, 10%
Mn ²⁺	1, 5, 10, 100 mM	poor, none at 100 mM	lobed cells, 10%
Methylamine-HCl	200 mM	good	lobed cells, 50%
Dimethylamine-HCl	150 mM	good	lobed cells, 30%
Trimethylamine-HCl	100 mM	fair	lobed cells, 30%
Ethanolamine-HCl	40 mM	none	—

N.B. It is not unusual to see 1-5% lobed cells in a culture from late exponential phase onwards.

results showed that, unlike Geodermatophilus, these compounds did not significantly affect the morphology of Hyphomicrobium (Table 3.3). However, the medium became very acidic with the amine compounds, possible reflecting changes in the intracellular pH. Zarlengo and Abrams (1963) suggest that a change in intracellular pH can have a profound influence on the metabolism of an organism. In their studies on S. faecalis, glycolysis was inhibited by a low intracellular pH, but the uptake of NH_3 or organic amines restored glycolysis immediately. Inorganic cations and organic amines were shown to raise the intracellular pH in Geodermatophilus, by Ishiguro and Wolfe, and this could be a possible explanation for what is occurring in Hyphomicrobium when the carbon source is switched from methanol to methylamine. Continuous incubation of the cells with methylamine after several subcultures ultimately resulted in a loss of lobed cells from the cell population, possibly due to a gradual return of the cell to its 'correct' pH level, due to ion exchange through the cell membrane.

Ultrastructure of lobed cells

Ultrathin sections cut through lobed cells revealed that they were similar to normal cells, DNA and cytoplasmic material comprising the body of the cell (Fig. 3.48). Again no intracellular membrane system was observed, as has been shown for 'normal' Hyphomicrobium (Conti and Hirsch, 1965), however extensive compartmentalisation was apparent in isolated lobed cells and lobed cells with stalk outgrowths (Fig. 3.48). There did not appear to be any significant alteration in the composition of these cells, compared to the 'normal' cell type, and thus one can assume that they do not represent a resting cell type and that their phenotypic variation is one of morphogenesis in response, either directly or indirectly, to some environmental factor.

(b) Nitrogen variation and cellular expression

It has been indicated that the nitrogen source affects the growth and morphology of Hyphomicrobium (Hirsch and Conti, 1946b).

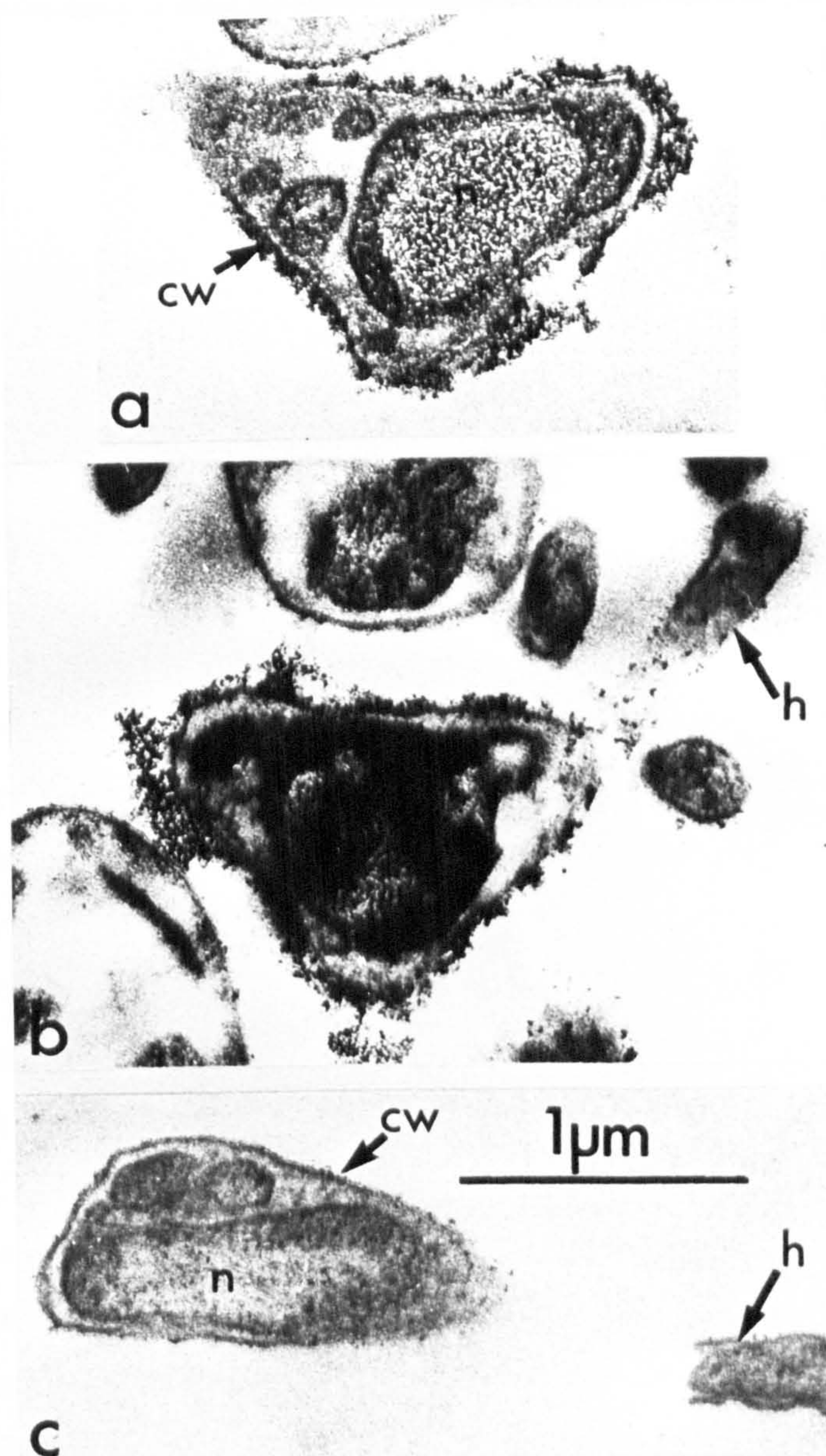


Fig. 3.48 Ultrathin sections of lobed cells. (a) lobed cell showing internal compartmentalisation, (b) stalk from a lobed cell, (c) presumptive lobed bud. No intracytoplasmic membrane system was observed .
 (cw = cell wall, n = nuclear material, h = hypha or prostheca)

$(\text{NH}_4)_2\text{SO}_4$ was routinely used as a source of fixed nitrogen, and appeared to support classical cell growth and cell morphology. A variety of nitrogen sources were tested for their ability to support growth of Hyphomicrobium and their effect on cellular morphology. Table 3.4 shows some of the results obtained from microscopic studies of cell cultures. All cultures were grown for five days, with the exception of formamide and acetamide which required twelve days for comparable growth. These studies clearly indicated that the nitrogen source could cause pleomorphism in Hyphomicrobium. This involved branching of the stalks, the production of stalks from several points on the cell surface, and sessile budding (Figs. 3.49, 3.50). Occasionally swellings were observed along the stalk length. Acetamide and formamide appeared to be taken up by the cells as a nitrogen source, causing bizarre cell shapes and sessile budding to occur (Fig. 3.8, 3.51 a-c).

Nitrite and nitrate, as sole nitrogen source, were readily utilised by Hyphomicrobium, being reduced to dinitrogen (Sperl and Hoare, 1971). Nitrate is normally included in the medium at 0.2% (w/v) when cultures are grown anaerobically (Attwood and Harder, 1972). At this concentration of NO_3^- , with ammonium sulphate also present at 0.5% (w/v), (see methods, 3.II.2.), the cell morphology appeared classical. Upon increasing the concentration of nitrate in the medium to 2% (w/v), the stalks became branched and bifurcated, and the buds when formed remained attached to the stalk, forming clusters (Figs. 3.50, 3.52). Above 2% (w/v), nitrate appeared to drastically inhibit growth either directly or through a product of its metabolism. Similarly, with nitrite, which could also be used as an artificial electron acceptor, there was considerable pleomorphism within the cell population (Fig. 3.51d-f). Nitrite appeared to retard growth above a concentration of 1.5% (w/v) although the cells remained viable (Table 3.4).

Table 3.4 The effect of nitrogen source variations on the growth and morphology of Hyphomicrobium

<u>Nitrogen source</u>	<u>Growth</u>	<u>Concentrations used</u>	<u>Cell morphology</u>
$(\text{NH}_4)_2\text{SO}_4$	good	0.05%	classical cell type
$(\text{NH}_4)_2\text{HPO}_4$	good	0.05%	classical cell type
Methylamine hydrochloride			
$\text{CH}_3\text{NH}_2 \cdot \text{HCl}$	good	200 mM	some lobed cells
NO_2^*	good	0.1-1.5%	classical cell types. 3 cell chains
NO_3^*	good	0.1-2.0%	much branching; sessile budding
HCONH_2	poor	0.2%	bizarre cell shapes; sessile budding
CH_3CONH_2	poor	0.2%	sessile budding

Good growth = ≥ 1 mg/ml protein within five days from culture inoculation.

* These compounds were assayed quantitatively (see Section 3.II.6).

Gas production was also assayed (Section 3.II.8).

Studies were carried out aerobically and anaerobically.

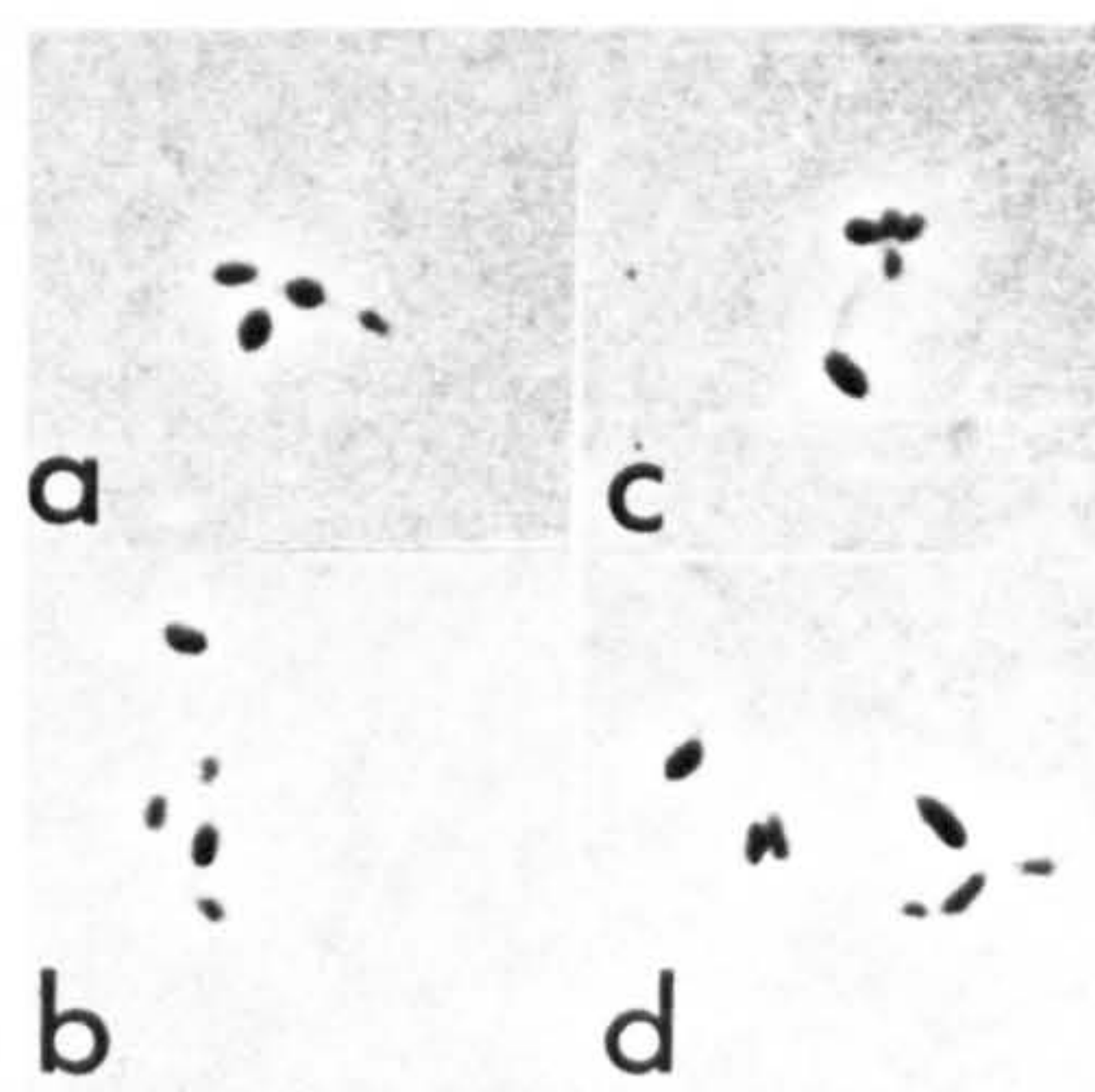


Fig. 3.49 Nitrogen source variations - effect on cellular morphology. Growth in the presence of 1.0% NO_2^- , cells became branched. (Magnification as Fig. 3.50).

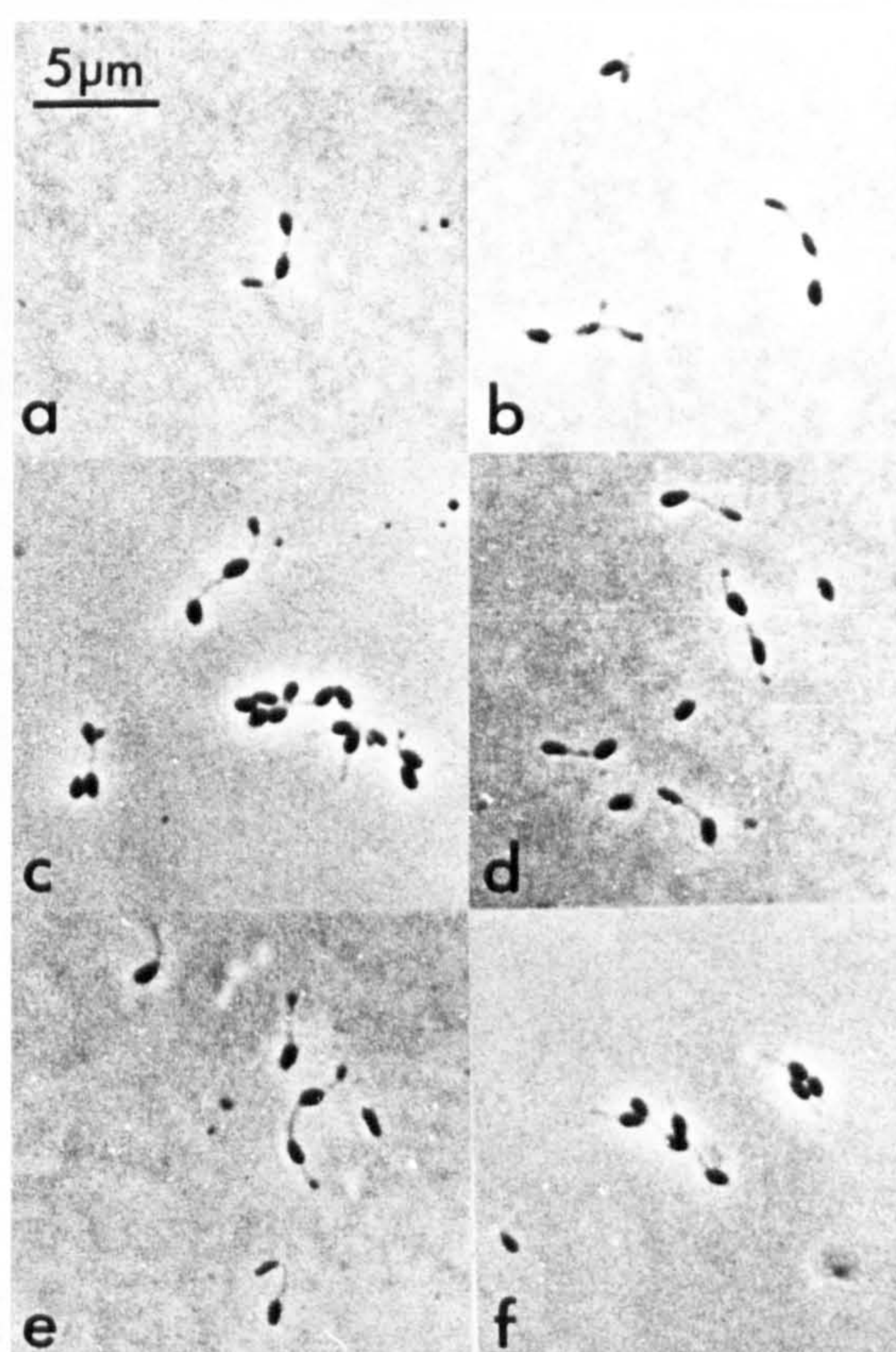


Fig. 3.50 Growth on NO_3^- . *Hyphomicrobium* became branched and complexed as multicellular arrays, as the nitrate level was increased. (a), (b), 1% (w/v) KNO_3 ; (c), (d), 1.5% (w/v) KNO_3 ; (e), (f), 2.0% (w/v) KNO_3 .

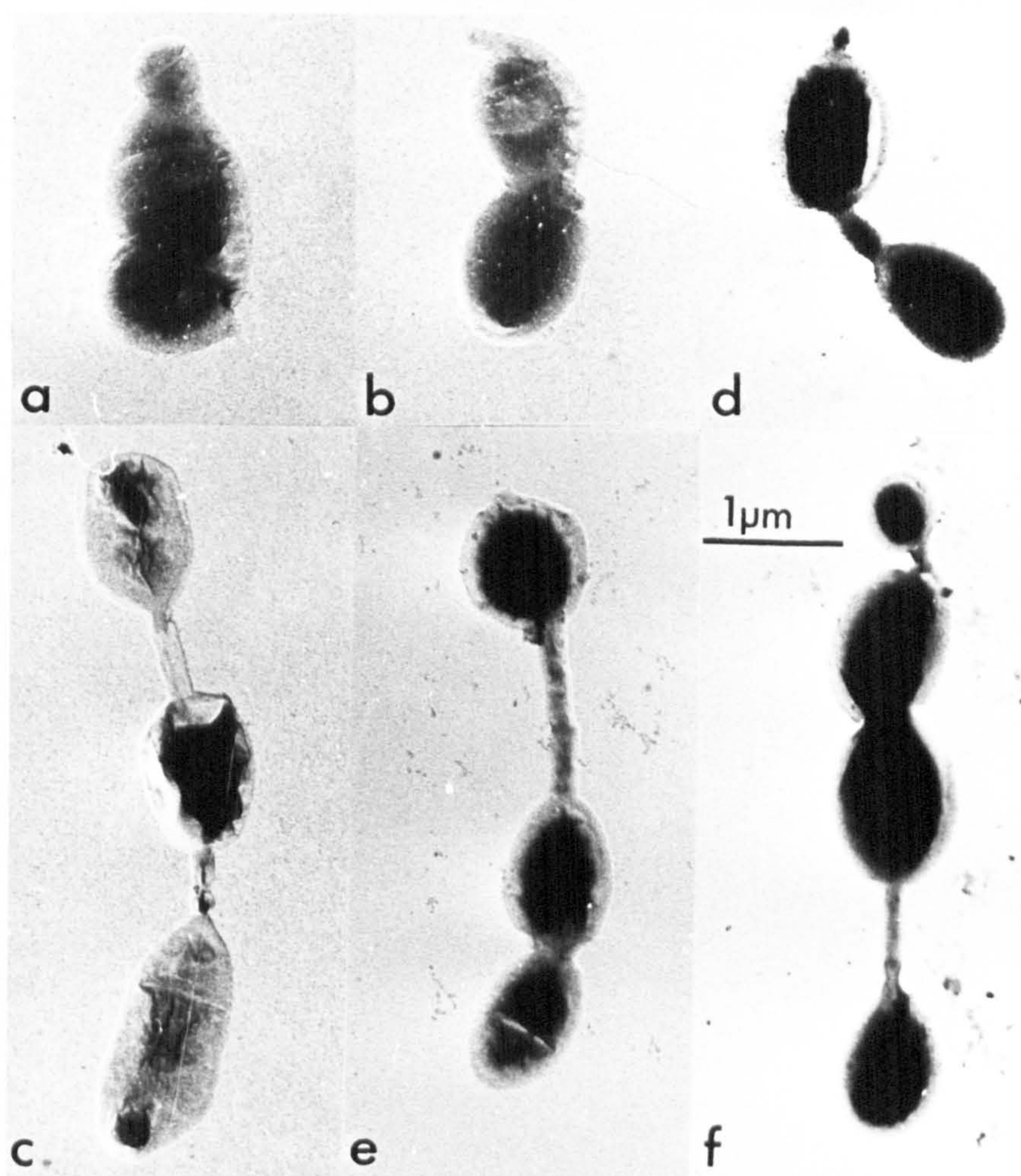


Fig. 3.51 Electron micrographs of Hyphomicrobium grown on (a) formamide, (b), (c) acetamide and (d), (e), (f) nitrite. Sessile budding occurred and stalks became very irregular, rarely performing their characteristic reproductive function of bearing buds (f). (Gold/Palladium shadowed).

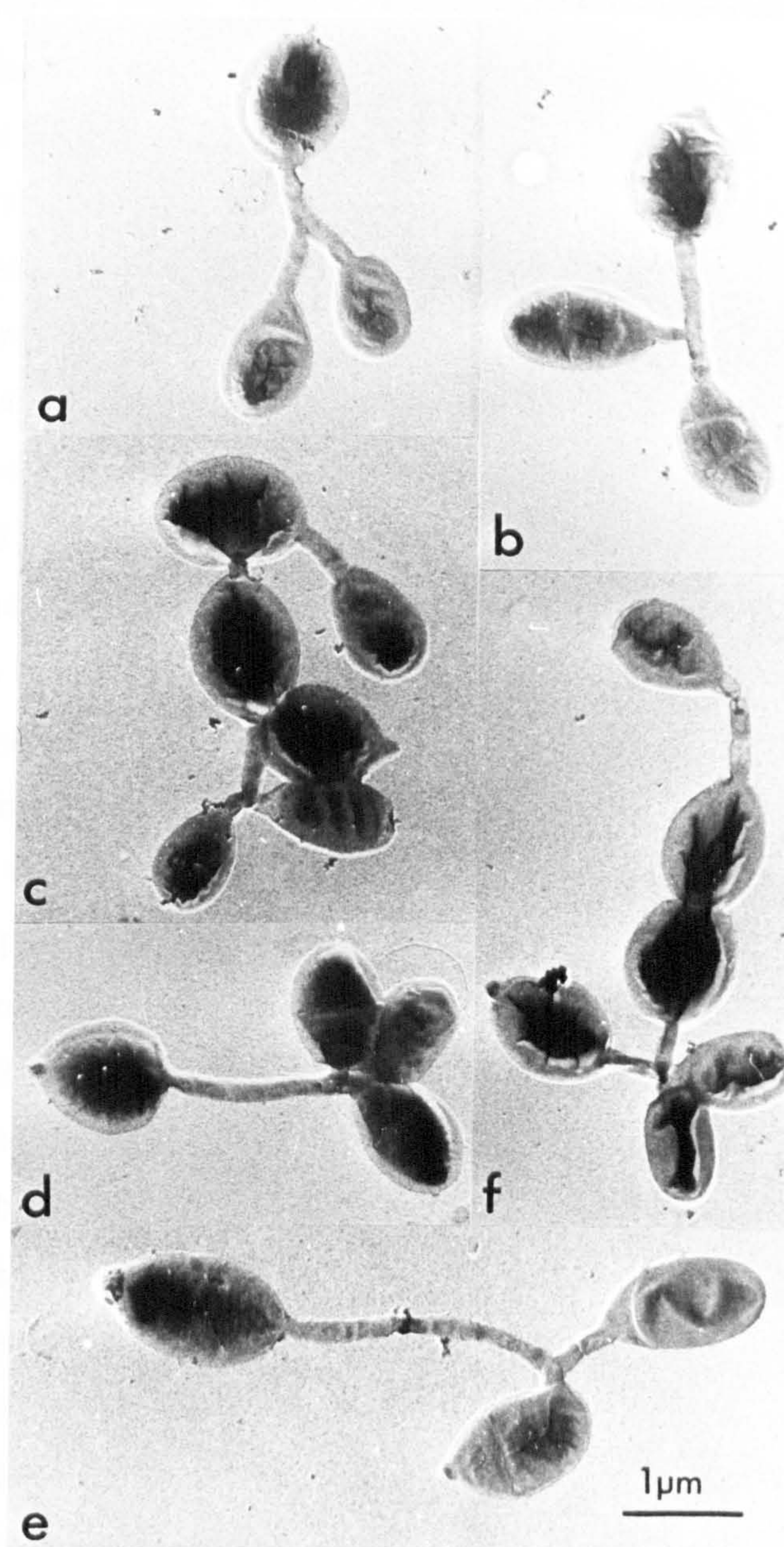


Fig. 3.52 Effects of increasing concentrations of NO_3^- , as source of fixed nitrogen on the morphology of *Hyphomicrobium*. (a) and (b), 1% (w/v) NO_3^- , (c) and (d), 1.5% (w/v) NO_3^- , (e) and (f), 2% (w/v) NO_3^- . Up to 2% (w/v) NO_3^- growth appeared normal. (Gold/Palladium shadowed).

(c) The effects of metalsIntroduction

Considering the studies of Aristovskya (1961, 1963), Hirsch and Conti (1964), Zavarzin (1964, 1968), Tyler and Marshall (1967) concerning the effects of heavy metals, manganese and iron and their oxidation states on the morphology of Hyphomicrobium, further investigations were undertaken. The ability of some microorganisms to oxidise dissolved manganous salts, and to precipitate them has been known for many years, however until 1960 there had been no direct observations of manganese oxidising bacteria, which in the natural environment are camouflaged by the accumulation of heavy metal oxides. (Aristovskya (1958) and Zavarzin (1960) used a pedoscope, which enables direct observations of microorganisms in their natural environment, to show that bacteria do accumulate heavy metals. They proposed that some bacteria utilize organic substances, complexed with heavy metals, which subsequently accumulate as waste products, however it was never demonstrated that they derive energy from this process. Other bacteria, observed with the pedoscope, accumulated manganese and iron specifically. This latter group of organisms included Pedomicrobium and Metallogenium (Aristovskya, 1963; Zavarzin, 1964). Studies by Tyler and Marshall (1967) on the microbial oxidation of manganese in hydroelectric pipelines showed that prosthecate, budding bacteria are capable of manganese deposition. They showed that a lack of deposition was due to low metal concentrations and not to the absence of the appropriate microorganisms. Before this study (Tyler and Marshall, 1967a), the sheathed 'iron bacteria' were assumed to be responsible for manganese deposition, but these workers demonstrated that stalked bacteria, subsequently shown to be Hyphomicrobium, were of greater significance.

Similarly, it has been known for some years that iron deposition can occur as a result of the breakdown of organomineral humus complexes by microorganisms (Aristovskya, 1961). Hirsch (1967) has shown that budding bacteria from aquatic or terrestrial habitats accumulate ferric hydroxide. The ferric hydroxide deposition was found to be initiated at 'primary active sites' on the bacterial cell surface, and eventually the

stalks and cell bodies became completely encased by a heavy coat of this deposit. Hirsch showed that the bacteria produced multiple stalks from rod-shaped cells which closely resembled Pedomicrobium.

The use of metal clips as a source of iron was derived from Hirsch (1968) who observed their corrosion and the subsequent deposition of iron after a few weeks. Some of these deposits contained hyphomicrobia. If the deposits were subcultured into fresh medium, after three months a thick pellicle covered the surface of the culture medium, and heavy deposits were evident at the bottom of the culture flask; both pellicle and sediment were shown to contain stalked budding bacteria (Hirsch, 1968). Shah and Bhat (1971) showed similar results, isolating hyphomicrobia from rusty nodules developing on an iron wire immersed in bore well water.

Results and Discussion

The following studies were carried out to determine the role of prosthecate bacteria in heavy metal depositions in the natural environment, and to characterise the organisms involved.

Observation and isolation of manganese and iron depositing bacteria

After several weeks a pellicle was formed on manganese rich medium which, when streaked on to MnSO_4 agar plates, yielded small dark brown colonies, the examination of which revealed a stalked bacterium (cf. Section 2.III.2). Acid treatment was required to show by microscopy that the cells were ovoid and the stalks repeatedly branched, forming an extensive network (Figs. 3.53 a-d, 3.55). Tyler and Marshall (1967a) isolated a stalked budding bacterium from manganese deposits which they showed to be a Hyphomicrobium sp., although certain pleomorphic forms closely resembled Pedomicrobium as described by Aristovskya (1961) (see Section 2.III.2). If the colonies isolated in this study were inoculated into liquid media, they eventually acquired the classical morphology of Hyphomicrobium; however, on solid media the cells maintained their pleomorphism together with their ability to deposit manganese in the medium.

Studies carried out using iron enrichments resulted in light brown pellicles forming within 3 weeks, which when plated

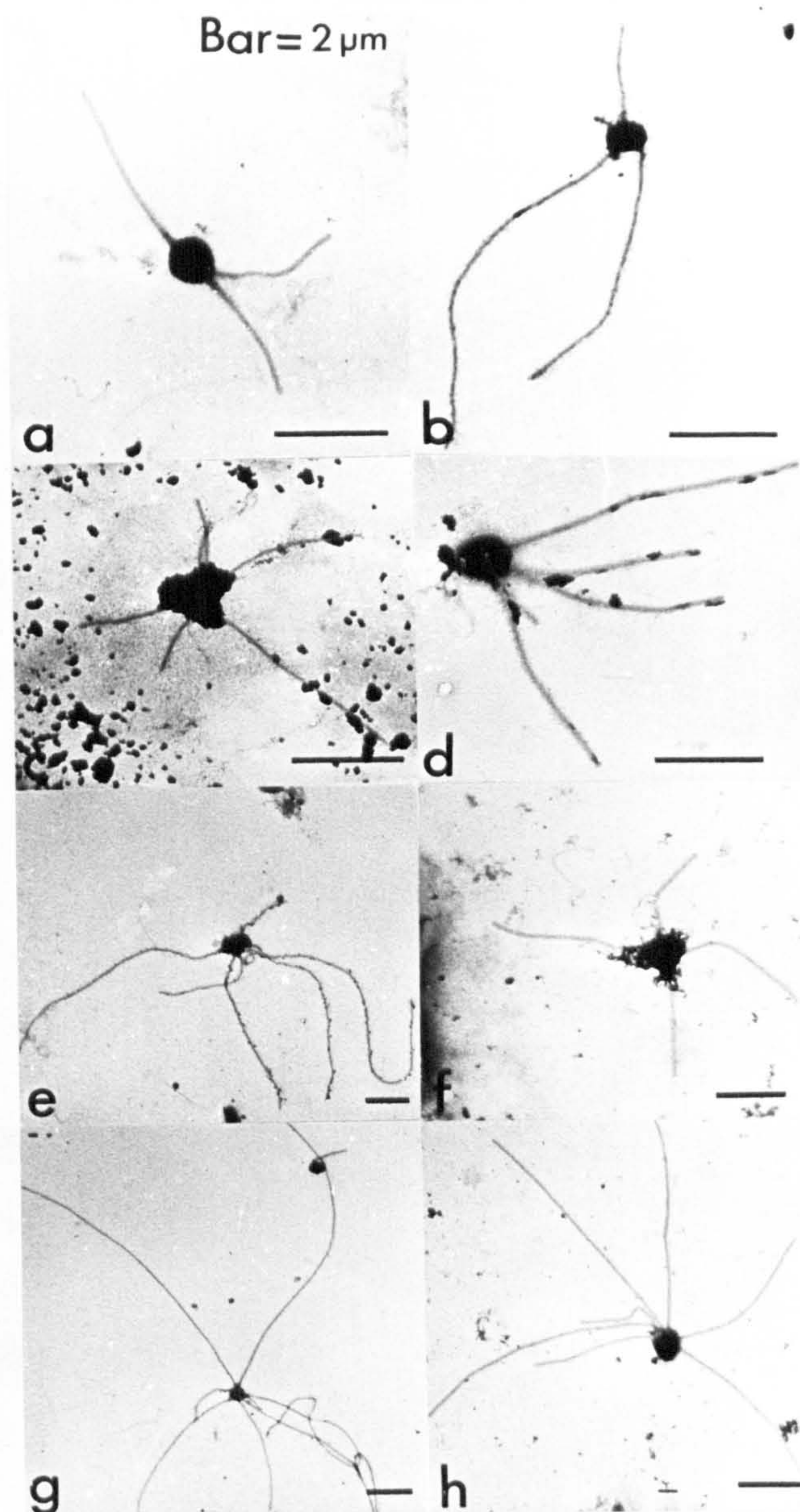


Fig. 3.53 'Static' enrichments after 3 months' incubation. Effects of heavy metals. (a)-(d) manganese enrichments, (e)-(h) iron enrichments (details in Text). Cells developed several prosthecae, emerging from several locations on the cell body which were occasionally masked by metal deposits (c) and (f). Prosthecae were long in comparison to cell body, and rarely branched or bifurcated.

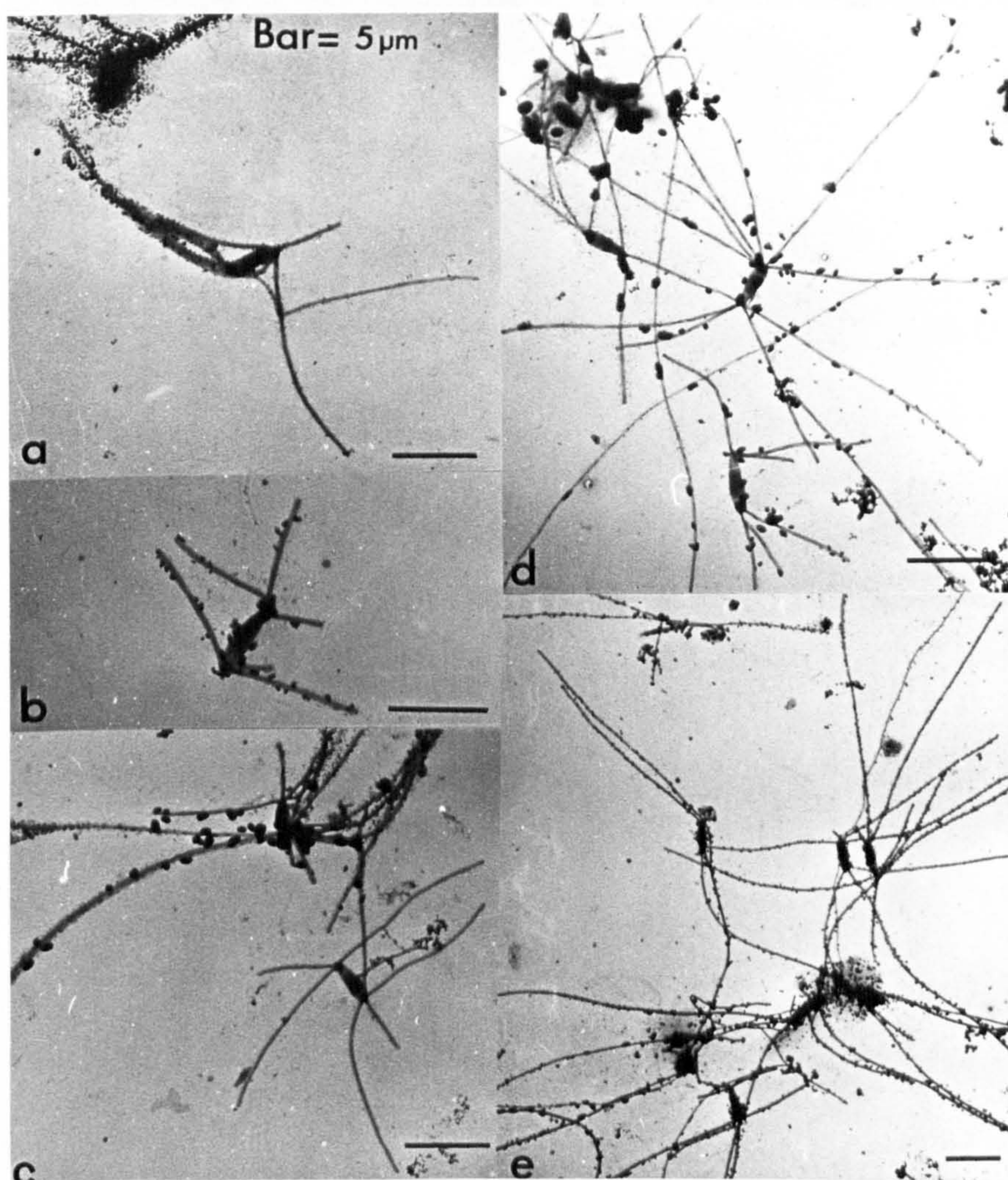


Fig. 3.54 Iron enrichments after 6 months 'static' incubation. Multi-appendaged cells and cellular arrays had developed. Usually the prosthecae extended from the poles, several emerging from one pole (d). Deposits of metal hydroxides formed on the cell body and appendages (b) and (c).

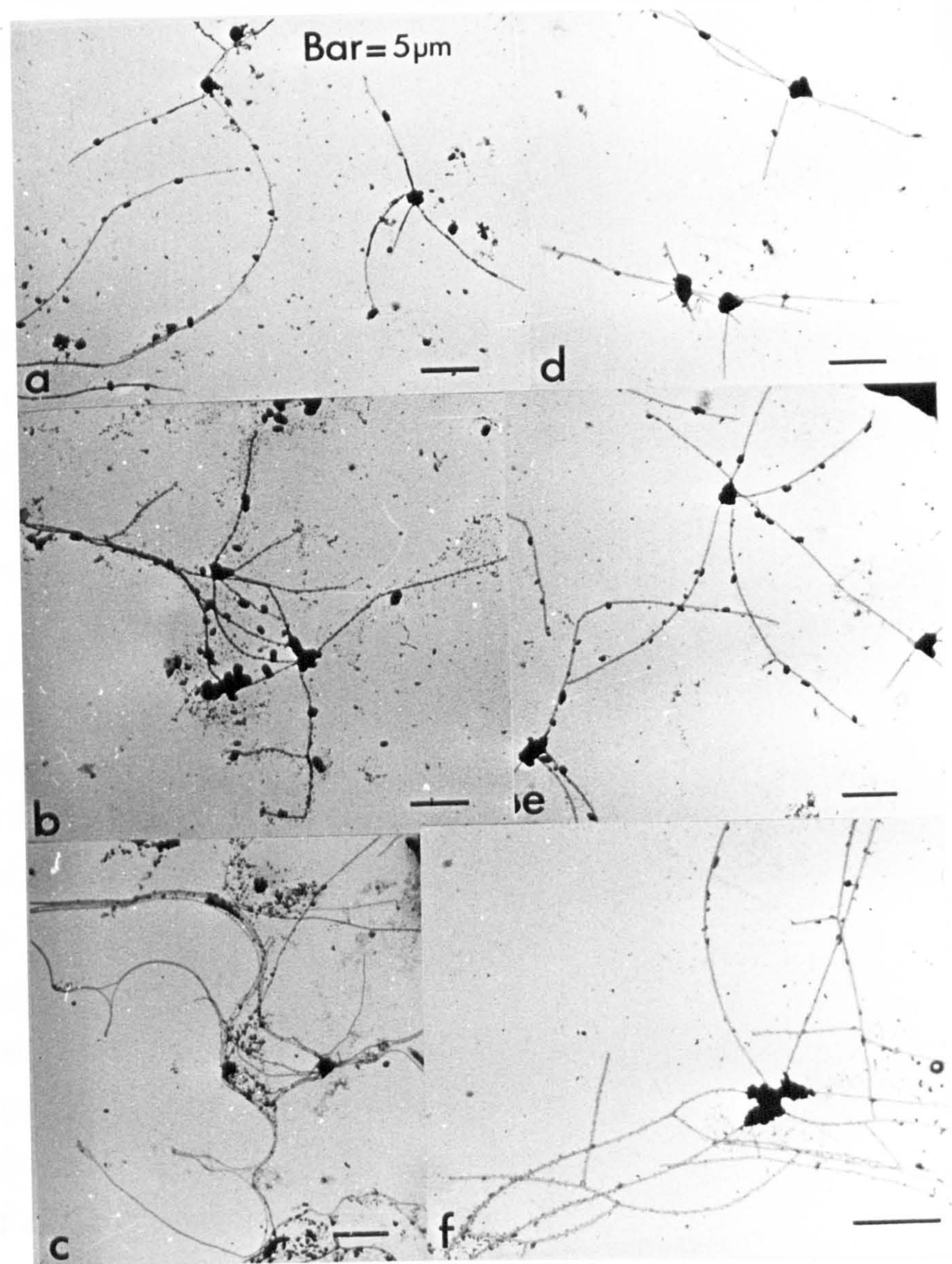


Fig. 3.55 Manganese enrichments after 6 months 'static' enrichment. Multiappendaged cells, characteristically resembling 'Pedomicrobium' developed (d), as individual cells (a) or as part of multicellular arrays (b, c and f). Metal deposits formed about the cell body and prosthecae (b).

out on solid media had the characteristics of Pedomicrobium (Fig. 3.53) (Section 2.III.2), however after several subcultures into liquid media, the culture became buff-coloured, with the cells appearing to morphologically resemble Hyphomicrobium, although some still appeared to retain some deposition of iron oxides.

Isolates of stalked budding bacteria, from both manganese and iron enrichments, were obtained from the pellicles that eventually formed on the surfaces of the liquid enrichments, and appeared to have characteristics of both Hyphomicrobium and Pedomicrobium, depending upon the concentrations of metals present. It appeared necessary to compare these results with a study of the effect of these heavy metals on Hyphomicrobium isolates, in order to determine whether Pedomicrobium was but a phenotypic expression of Hyphomicrobium under certain environmental conditions.

The effect of heavy metals on the morphology of Hyphomicrobium

Pure isolates of Hyphomicrobium and 'metal depositing' stalked cells were grown up in HB medium supplemented with iron or manganese salts, as described in Methods, Section 3.II.13. Rhodopseudomonas palustris was used as a control, being a prosthecate budding bacterium ubiquitous to freshwater, which is not known to deposit metal oxides. Table 3.5 shows that Hyphomicrobium tolerates high levels of these heavy metals, although above 5 mM, they were bacteriocidal. It was interesting to note that at concentrations where clumping of cells due to early metal deposition was not noticeable, the morphology of the cells was already altering (Fig. 3.56a) with 1 mM iron or manganese, growth was still reasonable ($O.D_{540} = 0.8$), but branching of the stalks was extensive, and cells became encased in metal deposits within two days (Fig. 3.56c). Above a concentration of 5 mM, few free cells could be observed, under phase contrast microscopy. R. palustris did not grow in metal concentrations above 1 mM, added to PAYE medium (Westmacott and Primrose, 1976). Below 1 mM the cells did grow, but poorly; they displayed no ability to deposit the heavy metals, and showed classical morphology, although the cells elongated in 1 mM $FeSO_4 \cdot 7H_2O$ supplemented medium.

Table 3.5 Growth of *Hyphomicrobium* in the presence of heavy metals

Mn as $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ metal salt concn.	aerobic		anaerobic	
	growth	morphology	growth	morphology
100 μM	++++	p/s, motile	++++	p/s, motile
500 μM	++++	p/s, motile	++++	p/s, motile branching
1 mM	+++	p/s, motile, branching, some clumping	+++	p/s, motile branching
2 mM	+++	p/s, motile, branching, some clumping	+++	extensive branching, clumping
5 mM	++	matrix, cells bound rarely free	+	matrix, PHB high in cells
10 mM	+	few cells observed without acid treatment	+	matrix, PHB high in cells

Fe as $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ metal salt concn.	aerobic		anaerobic	
	growth	morphology	growth	morphology
100 μM	++++	p/s, motile	++++	p/s, motile
500 μM	+++	p/s, motile	++++	p/s, motile
1 mM	+++	branching, chains	++	p/s, motile
2 mM	+++	clumping, much branching	++	extensive branching; chain forms
5 mM	+++	matrix, still a few motile cells	+	clumping and matrix
10 mM	++	solid matrix	+	clumping and matrix

NOTES

1. Growth as a positive response, assuming against control - no added Mn/Fe
++++ $\text{O.D}_{540} = 1.5$
2. Normal morphology = pairs/swarmers (p/s), motility, no clumping.
3. These results are mean for eight isolates studied.

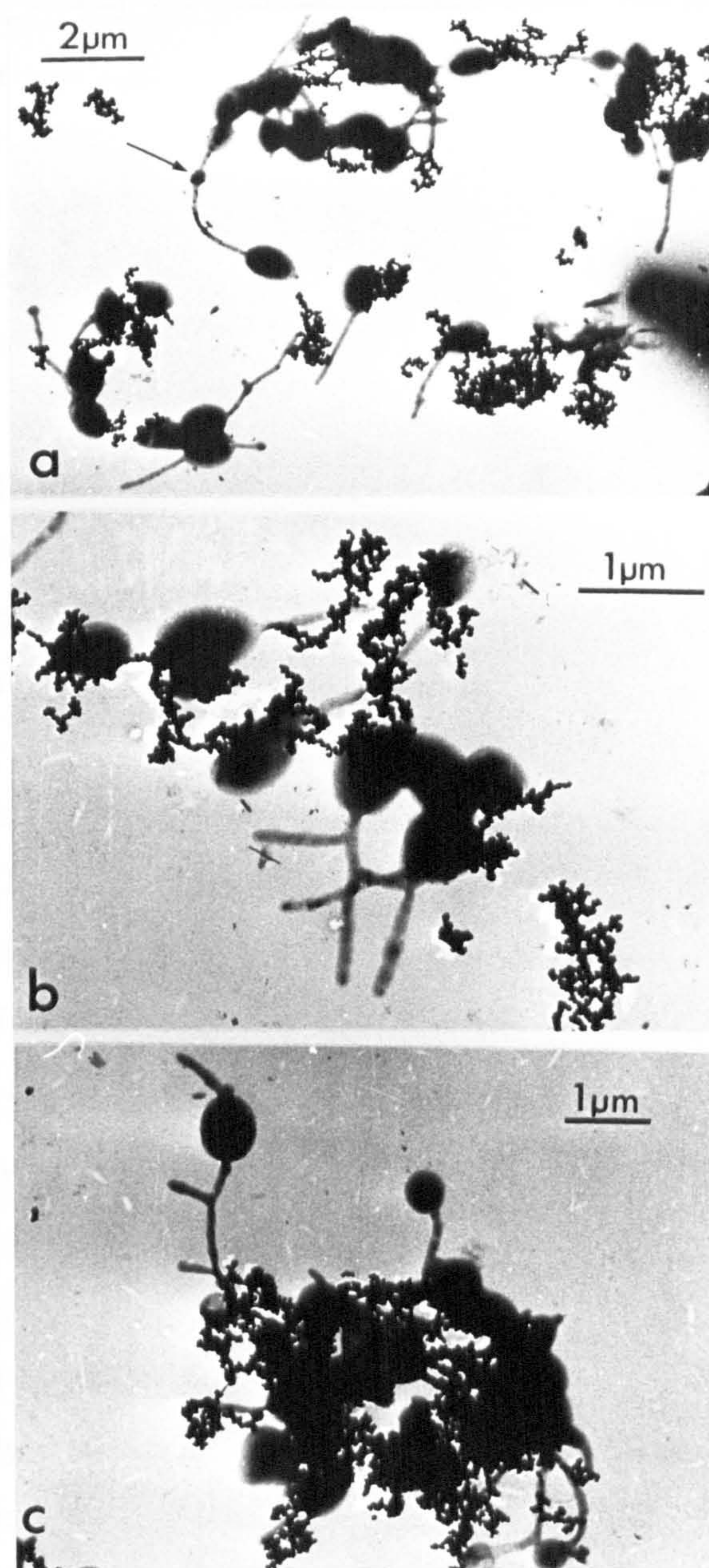


Fig. 3.56 Hyphomicrobium growing in manganese supplemented salts medium. (a) 500 μM Mn, (b) 1 mM Mn, (c) 2 mM Mn. Although the cells grew, they soon became pleomorphic and deposited metal hydroxides. Arrow in (a) indicates an intercalary bud. In (c) few cells are free, and those that are have branched appendages.

With all cultures, the morphology of the cells was similar in the presence of iron or manganese (cf. Figs. 3.53, 3.54, 3.55). No one isolate deposited iron and manganese oxides; only one of the eight isolates tested utilised manganese salts. There is no evidence for these cells utilising the heavy metals chemolithotrophically, under the culture conditions described (Tyler, 1970; Hirsch, 1974). Deposition of metal oxides initially started at a few sites on the surface of the cells, and the stalks, described by Hirsch (1968) as 'primary active sites'. His hypothesis proposes that excretions from these 'weak' areas of the cell wall result in local changes of the cell surface pH, probably towards alkalinity, as in the excretion of NH_3 during autolytic processes, which could initiate the oxidation of the metals. Hirsch proposed that the ferrous ions would be oxidised, and then further non-biological oxidation would deposit ferric hydroxides to give heavy deposits over the cell surface and stalks. This mechanism for iron deposition may well extend to manganese deposition. The subjection of Hyphomicrobium cultures to heavy metal salts resulted in considerable pleomorphism within the cell population, the cell forms closely resembling Pedomicrobium (Aristovskya, 1961).

Pleomorphism - morphological variation in a bacterial species

Zavarzin (1961) suggested a relationship between Hyphomicrobium, Rhodomicrobium and Pedomicrobium. Hyphomicrobium, depending upon cultural or environmental conditions, may develop multistalks and eventually multicellular arrays, as have been described here, supporting the observations made by Tyler and Marshall (1967a) on their isolate of Hyphomicrobium, designated T37, thereby closely resembling Pedomicrobium (Aristovskya, 1961). T37, when grown on minimal media 337 (Hirsch and Conti, 1964a) with methanol and 0.02% (w/v) $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, appeared typically like Hyphomicrobium vulgare, the type species, however when grown on Pringsheim medium (Pringsheim, 1949) with 0.002% (w/v) $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ and

0.005% (w/v) yeast extract, T37 underwent extensive branching to closely resemble Pedomicrobium. The studies carried out in this project support the observations made by Tyler and Marshall (1967b). Pleomorphism in Hyphomicrobium clearly is dependent upon cultural conditions, e.g. the presence or absence of heavy metals, however cultural conditions do not necessarily reflect the natural environment, and so care must be taken when extending the observations made in the laboratory to how the bacteria may exist in nature.

Role of Hyphomicrobium in heavy metal deposition

It would appear that the ability of Hyphomicrobium to accumulate iron or manganese deposits is accidental (Hirsch, 1968). The ecological implications of metal deposition for stalked, budding bacteria is quite clear. As the older parts of the cell become encased in these metal crusts, stalks grow out from the cell body to release swarmer cells beyond the confines of the metal encrusted complexes. Iron and manganese deposition appeared ubiquitous for all the isolates of Hyphomicrobium that were tested. Hirsch (1968) was unable to show this type of pleomorphism in all his strains of Hyphomicrobium and so consequently he maintained that Pedomicrobium was growing in these heavy metal deposits. However, as Aristovskya (1961) described the morphology of Pedomicrobium.... "from one to four threads grow out from a cell", even Pedomicrobium would not accommodate some of the morphological forms observed in iron or manganese supplemented cultures (Figs. 3.54, 3.55).

In the natural environment, Tyler and Marshall (1967a) showed that in hydroelectric pipelines, nutrient levels were low although the water was rich in manganese, and the flow rates were high. Any organism which could adhere to the pipes would be able to take advantage of the constant nutrient flow. Hyphomicrobia, with their holdfasts and secreted mucilaginous material (Marshall and Cruickshank, 1973) could adhere, withstanding the manganese deposits by their unusual reproductive cycle, to become predominant under such conditions.

(d) Pedomicrobium v. Hyphomicrobium

Pedomicrobium and Hyphomicrobium were considered with respect to:-

- (1) Morphology in the natural environment and pure culture.
- (2) Physiology.
- (3) G+ C% base ratios.
- (4) DNA homology.
- (5) Pathway for assimilation of carbon material into cell constituents.
- (6) Soluble protein patterns as determined by polyacrylamide gel electrophoresis,

in order to determine the validity of the genus Pedomicrobium. An isolate of mushroom-shaped bacterium, MpD₁ was used as a comparative study, being a budding bacterium present in oligotrophic environments.

(1) Morphology

Hyphomicrobium (see Section 3.III.2). The cells are ovoid with stalk(s) which may show true branching. Multiplication is by budding at the tips of the stalk(s). Pleomorphism, although well documented, is not considered in the genus description. (Tyler and Marshall, 1967; Hirsch, 1968; this thesis, Section 3.III.4).

Pedomicrobium (see Section 2.III.2). The cells are ovoid with one to numerous stalks extending from several sites on the cell body (Buchanan and Gibbons, 1974). Multiplication is by budding from the tips of the stalks; the buds may remain attached or may separate. Depositions of iron or manganese are frequently observed on the cell bodies and stalks.

(2) Physiology - nutrition and growth conditions

Both organisms appeared similar, being chemoorganotrophic with a preference for one carbon compounds (Table 3.6). Growth conditions for both organisms were also similar (Table 3.6).

Table 3.6 Hyphomicrobium v. Pedomicrobium.A comparison of genus characteristics

<u>Characteristics</u>	<u>Hyphomicrobium</u>	<u>Pedomicrobium</u>
(1) Cell morphology		
cell shape	round to ovoid, one or two stalks, from polar locations	round to ovoid, multi-stalked, from locations all over cell surface
motility	polar flagellum	polar flagellum
pleomorphism	lobed cells, multicellular arrays	multicellular arrays
isolation	oligotrophic lake/HB enrichment	oligotrophic lake/HB enrichment, with Fe supp.
(2) Colonial morphology	small, white and slightly mucoid, brownish with age	small, brownish in colour
(3) Gram stain	Gram negative	Gram negative
(4) Physiology		
nutrition	chemoorganotrophic - C ₁ compounds, a few C ₂ compounds ; can also grow oligo-carbophilically	chemoorganotrophic - C ₁ compounds
nutrient supplements	trace elements	trace elements; at least 0.005% Mn or Fe salts
temperature	15 ⁰ -45 ⁰ C	15 ⁰ - 45 ⁰ C
pH	neutral, slightly alkaline	neutral
aerobic <u>v.</u> anaerobic	aerobic	microaerophilic to aerobic
(5) Assimilation pathway for carbon material	serine	serine
(6) G + C % base ratios.	60.3	60.18
buoyant densities (g/cm ³)	1.7191	1.7190

(3) DNA - G + C % ratios

The DNA extracted from exponentially growing Hyphomicrobium H1 and Pedomicrobium P1 was subjected to neutral caesium chloride (CsCl) buoyant density centrifugation. Hyphomicrobium and Pedomicrobium had very similar densities and guanine plus cytosine base ratios (Table 3.6). The G + C % ratio of hyphomicrobia range from 59.2 to 66.8% (Hirsch, 1974) (Table 3.1).

(4) DNA base sequence homologies

The genetic relatedness of Hyphomicrobium H1 and Pedomicrobium P1, isolated during the course of this study, was determined by DNA homology experiments (Table 3.7). The extent of the cross reaction between the DNA of H1 and P1 was 69.0% and between the DNA of P1 and H1 was 67%, which when corrected for with respect to the homoduplexes were 83.75% and 82.51% respectively. This suggests a considerable degree of relatedness between the two organisms and indicates that their genomes are of equivalent sizes, as the results are the same on both reannealing combinations. Moore and Hirsch (1972) demonstrated a wide diversity in the base sequence of DNA species from various strains within the genus Hyphomicrobium, but with only a poor degree of base sequence homology existing. These authors carried out DNA association experiments between Hyphomicrobium and various other prosthecate bacteria, however heteroduplexes were not formed; Pedomicrobium was not tested.

(5) Pathway for assimilation of carbon material into cell constituents

Three pathways are known whereby the net synthesis of a three-carbon skeleton from one carbon compound is accomplished, the ribulose diphosphate cycle of carbon dioxide fixation, the ribulose monophosphate cycle of formaldehyde fixation and the serine pathway (Harder, 1977). The serine pathway has been shown to operate in Hyphomicrobium (Attwood and Harder, 1973) as the icl⁻ variation (Attwood, 1977), as described in Section 3.1. of this thesis.

^{32}P labelled DNA		"Cold" DNA	Cot.	% Reaction	% Reaction corrected
(1) a.	H. 1	H. 1	100	87.6%	83.1%
b.	H. 1	none	0	4.5%	—
c.	H. 1	P. 1	66	74.1%	69.6%
(2) a.	P. 1	P. 1	66	96.5%	81.2%
b.	P. 1	none	0	15.3%	—
c.	P. 1	H. 1	100	82.3%	67%

Table 3.7

DNA base sequence homology study of Hyphomicrobium (H1) against Pedomicrobium (P1)

Details of experimentation, Section 3.II. The Cot. values for P1/P1 cross was lower than H1/H1, as less DNA was used in the hybridisation experiment, H1 - 10 O.D. units/ml., P1 - 7.18 O.D. units/ml).

Hydroxypyruvate reductase (D-glycerate NAD^+ oxidoreductase E.C 1.1.1.29) has been shown to be a key enzyme in the serine pathway (Large and Quayle, 1963; Blackmore and Quayle, 1970), and so it was assayed for in soluble protein fractions of Pedomicrobium, using Hyphomicrobium as the standard, and the budding mushroom shaped bacteria as a comparative study, as shown in Table 3.8. The assay was performed at pH 6.5 as this gave optimal activity. This assay demonstrates that Pedomicrobium is utilising the serine pathway, and although the specific activity of the enzyme assayed is lower, the values are comparable to those obtained for Hyphomicrobium. The values for the specific activity of the enzyme, when assayed for mushroom shaped bacteria, demonstrated its presence, but at a low activity, possibly reflecting the poor growth of this organism on C-1 compounds.

(6) Soluble protein patterns (polyacrylamide gel electrophoresis)

Soluble protein fractions prepared from Hyphomicrobium and Pedomicrobium together with mushroom shaped bacterium isolate MpD_1 as a comparative study, were run on 10%-30% exponential polyacrylamide gels, as described in Section 3.II.28) Fig. 3.57 shows a comparative run of Hyphomicrobium and Pedomicrobium with standard markers. The pattern profiles appear very similar on the three one-carbon compounds tested, the intense band represents a protein(s) of m.w. 69,000. The double band at m.w. 54,000 ran at the position of ribulose 1,5 diphosphate carboxylase (Codd and Stewart, 1977), but was not identified as this enzyme (S. Taylor, personal communication). In contrast the mushroom shaped bacterium MpD_1 , although a budding oligotrophic bacterium which utilises one-carbon compounds, gave a different soluble protein profile (Fig. 3.58).

These results suggest that Hyphomicrobium and Pedomicrobium possess the same soluble protein profiles on different carbon compounds which have been shown to give optional growth (Section 3.III.1).

Organism	Substrate	Protein concn. mg/ml	Units/ml	Specific activity units/mg protein
<u>Hyphomicrobium</u>	CH ₃ OH	1.8	5.79	3.2
	CH ₃ NH ₂	2.4	11.58	4.825
	HCOO ⁻	1.5	4.8	3.2
<u>Pedomicrobium</u>	CH ₃ OH	3.8	9.65	2.6
	CH ₃ NH ₂	4.2	15.43	3.8
	HCOO ⁻	2.5	2.5	1.0
Mushroom-shaped bacteria	CH ₃ OH	1.0	1.93	1.93
	CH ₃ NH ₂	1.0	0.096	0.096
	HCOO ⁻	2.4	3.9	1.6

Table 3.8
Assay of hydroxypyruvate reductase in soluble cell extracts of
Hyphomicrobium, Pedomicrobium and mushroom-shaped bacteria.
(Details of procedure, Section 3.II.27).

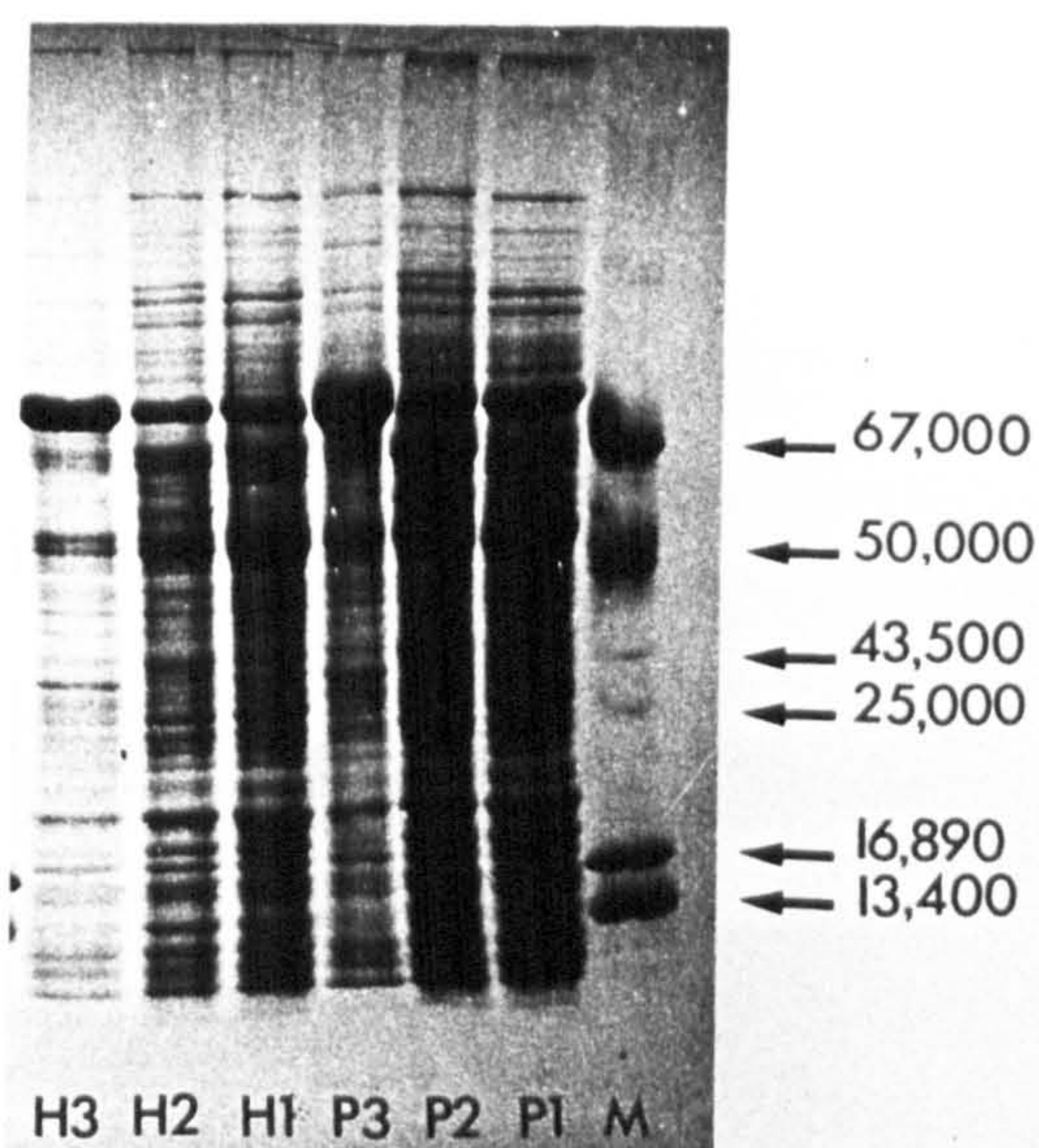


Fig. 3.57 10%-30% exponential polyacrylamide gel showing the protein pattern profiles of Hyphomicrobium (H) and Pedomicrobium (P) growing on (1) methanol, (2) methylamine and (3) formate, with standard molecular weight markers (see Method, 2. II).

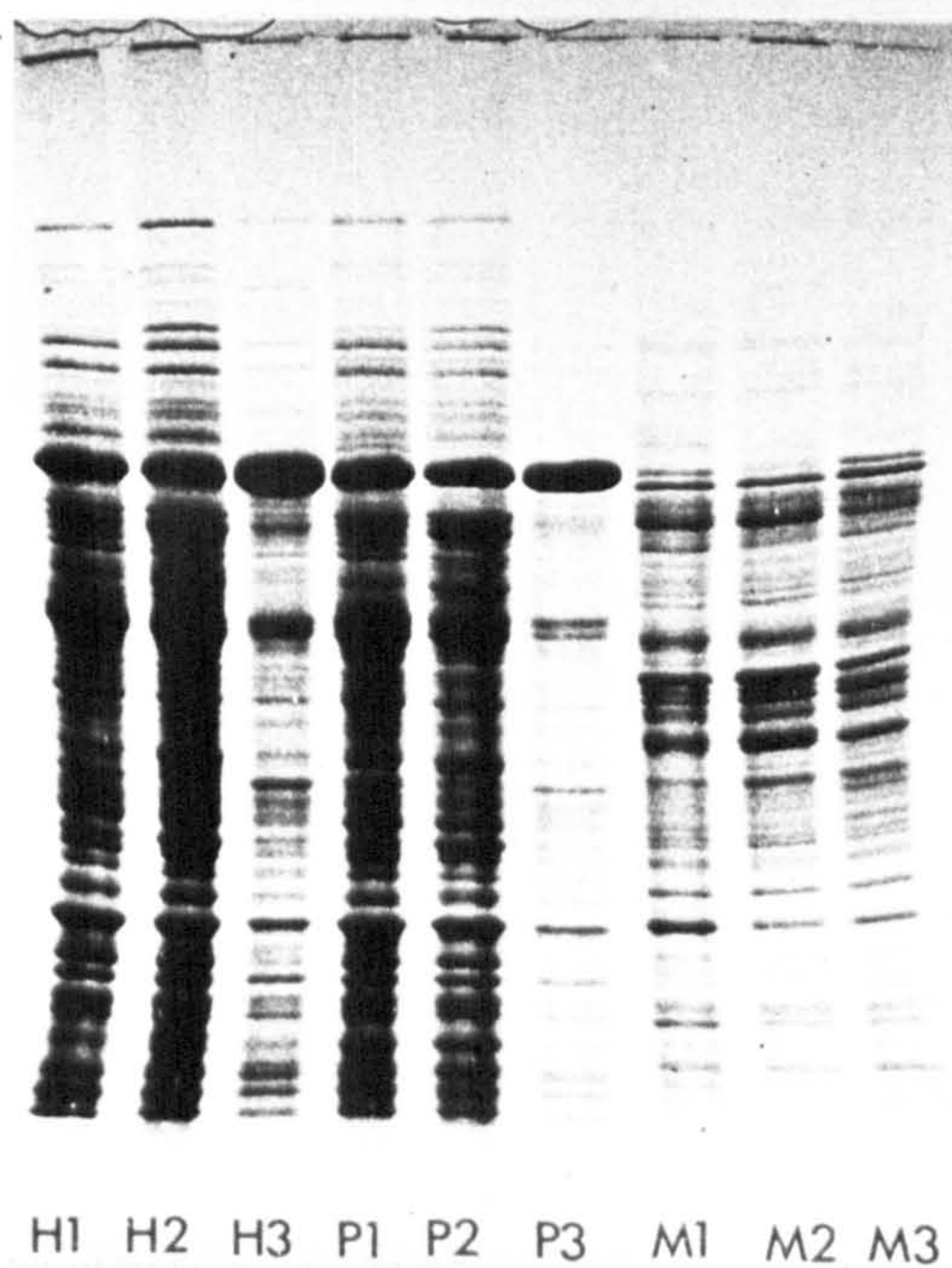


Fig. 3.58 10%-30% exponential polyacrylamide gel showing the protein pattern profiles of Hyphomicrobium (H), Pedomicrobium (P) and a mushroom shaped bacterium (M) grown on C-1 compounds.
(1) methanol, (2) methylamine, (3) formate.

Validity of the genus *Pedomicrobium*

Certain studies with regard to phenotypic variation in prosthecate bacteria have been cautious (Hirsch and Conti, 1964; Hirsch, 1968), classifying bacteria according to very inflexible generic descriptions, many of which were formulated before the electron microscope made morphological characterisation a routine procedure. Consequently, within certain groups of bacteria, phenotypic variants have been cited as new organisms and placed in new genera; the prosthecate bacteria represent one such group (Whittenbury and Dow, 1977). With regard to chemoorganotrophic prosthecate bacteria which possess prosthecae having a reproductive function, these are placed in one of three genera: Hyphomicrobium, Hyphomonas or Pedomicrobium. As has previously been discussed (Section 2.1), the criterion which has been used to distinguish between the genera Hyphomicrobium and Hyphomonas is that of DNA-DNA base sequence homology studies (Moore and Hirsch, 1972); however, as these authors demonstrate a wide range of values for different strains of Hyphomicrobium (1-100% homology), genus separation does not appear to be justified. Moore (1976) demonstrated that RNA cistron homologies among Hyphomicrobium and various other bacteria, resulted in an improved grouping, with Hyphomicrobium strains giving 72-100% homology, whereas Hyphomonas (Pongratz, 1957) gave 66% homology and Rhodomicrobium gave 55% homology. Pedomicrobium was not tested. Hirsch (1974) considers that H. neptunium (Leifson, 1964) should be removed from this genus and be placed in the genus Hyphomonas as they both have similar G+C% base composition (61.7% and 61.2% respectively) and similar DNA:DNA duplex homologies relative to the Hyphomicrobium standard employed. Hyphomonas appears unable to grow on C₁ compounds (Pongratz, 1957) whereas these compounds are the preferred, if not the only, carbon source for the growth of Hyphomicrobium spp. (Hirsch and Conti, 1964b; Sperl and Hoare, 1971; Attwood and Harder, 1974; cf. Hirsch, 1974).

Hyphomicrobium T 37 (Bauld, Tyler and Marshall, 1971) has been shown to exhibit extensive pleomorphism, which is dependent on the growth medium, and can assume the morphology previously described as characteristic of the genus Pedomicrobium (Aristovskya, 1961). Bauld et al. (1971) questioned the validity of the genus Pedomicrobium and suggested that this reflected one growth form of Hyphomicrobium. Harder and Attwood (1978) also cultured T 37 and demonstrated the same pleomorphism. They concluded that this organism cannot be accommodated in the genus Hyphomicrobium as defined (Hirsch, 1974) in Bergey's 8th Manual of Determinative Bacteriology, (ref. Buchanan and Gibbons, 1974).

The study undertaken clearly indicated that the presumptive Pedomicrobium resembled Hyphomicrobium both morphologically and physiologically, providing one considered phenotypic variation as acceptable in the definition of the genus. Base composition and DNA homology studies further indicated that these bacteria were very similar, which is remarkable when one considers the wide diversity in base composition experienced within the genus Hyphomicrobium. Both organisms utilised one-carbon compounds, preferentially by the serine pathway and their soluble protein profiles appeared very similar. Therefore, one must conclude that the genus Pedomicrobium is invalid, and that Pedomicrobium is a pleomorphic variant of Hyphomicrobium. Hyphomicrobium in pure laboratory culture therefore does not necessarily reflect the true morphological expression of this bacterium in the natural environment, where it may exist as individual cells or multicellular arrays, depending upon the nutrient status of the environment (Fig. 3.59).

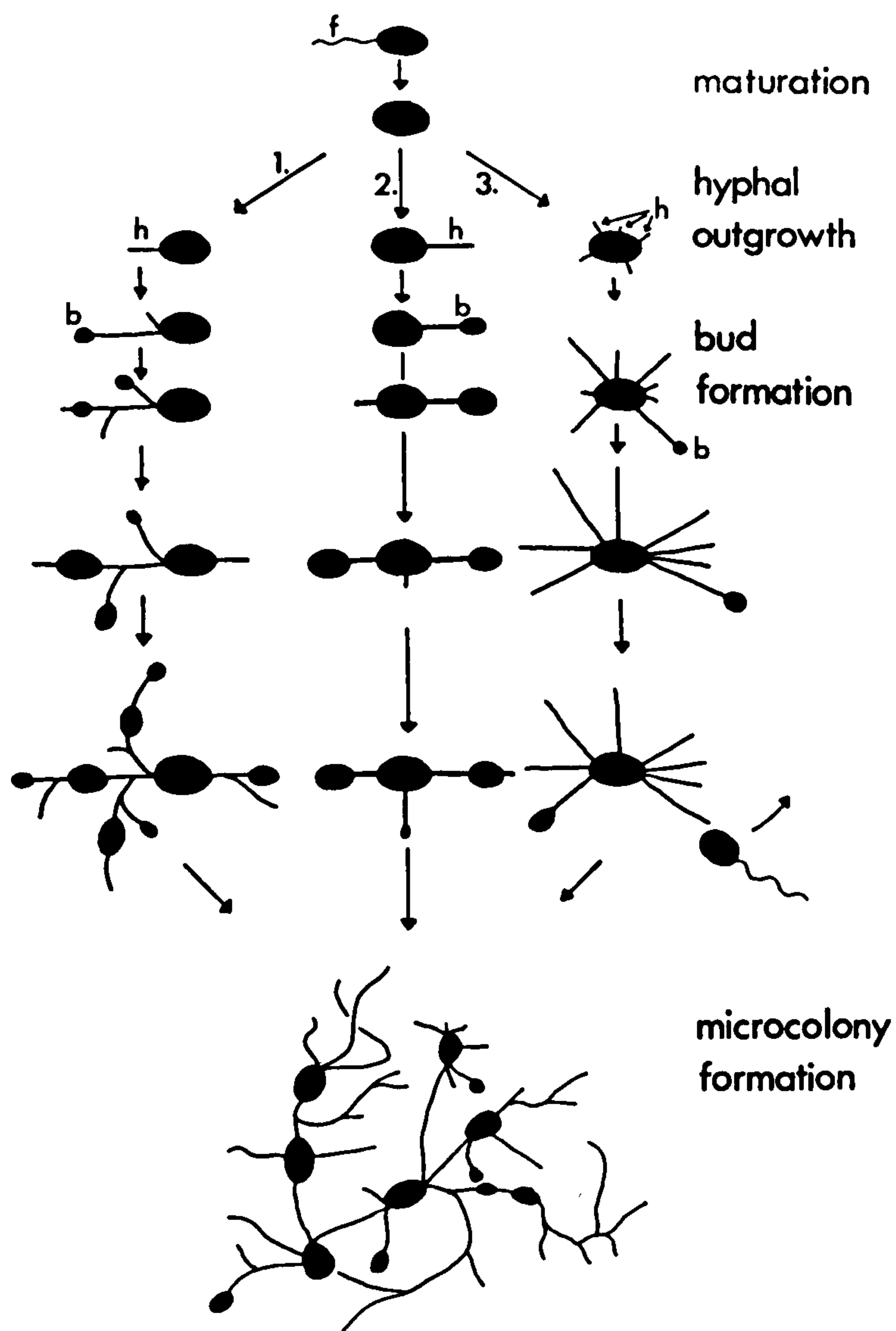


Fig. 3.59 Mode of development of *Hyphomicrobium*. Paths 1, 2 and 3 are observed in the natural environment, or when such conditions are artificially maintained in the laboratory, e.g. 'static' enrichments, with or without additions (metal salts). Laboratory culturing suppresses this pleomorphism. (h = hyphae or appendages, b = bud).

Section 4

Conclusions and Future Prospects

The budding prosthecae bacteria constitute a highly specialized population of cells, ubiquitous to freshwater environments.

'Enrichment' systems, to which very little or no nutrients were added, selected for these morphologically unusual bacteria, and several isolates were studied in detail. The adaptability of these stalked bacteria in nutrient poor environments demonstrated that their ability to vary their phenotype resulted in their dominance in such environments. As their presence is dependent upon low levels of nutrients, they are natural indicators of pollution. It is important to appreciate, however, that in these nutrient poor conditions, the maintenance energy (*i.e.* the energy required for the turnover of cellular constituents, the preservation of the right ionic composition and intracellular pH of the cell, and the maintenance of a pool of intracellular metabolites against a concentration gradient (Stouthamer, 1977)) required by these bacteria, by necessity must be lower than that required by E. coli. For example, at low growth rates, and consequently, under 'starvation' conditions, these bacteria are capable of surviving, whereas E. coli disappears from the cell population.

Of the bacteria which are capable of sustaining themselves in these nutrient poor conditions, only those possessing integral cellular extensions (termed prosthecae, and sometimes stalks, appendages, hyphae, filaments) or extracellular stalks maintained themselves in good numbers. The only organism possessing an extracellular stalk which was isolated from these freshwater enrichments was Planctomyces. Although the stalk of this organism is not directly involved in nutrient uptake, it does enable the cell to absorb nutrients from a wider field, and the network of fimbriae, which becomes extensive as the environment is 'starved' may well help to concentrate nutrients.

Bacteria possessing integral cellular extensions appeared to be capable of existing indefinitely in nutrient poor environments. There appeared to be a fundamental split concerning prosthecal function; those involved in reproduction which were consequently obligate, and those which were non-obligate and environmentally induced.

Hyphomicrobium, with its dimorphic life cycle, appeared to dominate nutrient poor environments, although under these conditions the cells became pleomorphic, many exhibiting multicellular forms, with extensive prosthecae extending from several locations on the cell body. This environmentally induced phenotypic variation would appear to invalidate the genus Pedomicrobium. The ability of Hyphomicrobium to deposit metal hydroxides also questions the validity of the genus Metallogenium. Although the stalk of Caulobacter does not bear the daughter cells, it is intimately involved in the life cycle, increasing in length with each successive cell cycle of the mother cell. In contrast, the expression of appendages (prosthecae) of the multiappendaged bacteria appears to be under environmental control, their induction or repression being a direct consequence of the nutrient status of the environment.

These studies highlight the possible functions of the prostheca. Prosthecal formation can be obligate, e.g. Hyphomicrobium or environmentally induced, e.g. Ancalomicrobium. In both cases, however, under nutrient poor conditions, the prosthecae increase in number and length, thereby increasing the surface area to volume ratio of the cell to optimise nutrient uptake. Under such growth conditions, uptake of nutrients to maintain the cells in such an environment is the primary function of the prosthecate bacteria, reproduction only being carried out when the conditions are more favourable. Similarly, in laboratory culture, swarmer cell development in Hyphomicrobium has been shown to be under environmental control, with the swarmer cells 'held' in development until environmental conditions become favourable for the development of the swarmer cell, i.e. this obligate temporal sequence, once initiated, must be carried through to its conclusions - a reproductive cell. Similarly, the swarmer cells of Caulobacter and Rhodomicrobium will only develop when environmental conditions are favourable.

These studies have shown that the prosthecate bacteria are the most adaptive microorganisms in oligotrophic waters, by reason of their ability to vary their phenotype as part of their obligate life cycle, e.g. Hyphomicrobium, Caulobacter, Planctomyces, and to adapt their morphological expression in response to the environment e.g. Hyphomicrobium, Ancalomicrobium. Most of these bacteria reproduce by asymmetric polar growth (budding), giving them the potential to evolve their morphology in response to environment constraints.

Preliminary studies were carried out to determine how the environment can initiate such morphological changes in these bacteria, by altering the growth medium and various physical parameters. Continuous culture studies have indicated that the growth rate affects the morphology of the cells. Further studies are needed in this area to determine whether 'energy' levels within these cells do 'govern' their morphology, e.g. by following the endogenous levels of nucleotide triphosphate pools through the development of the cell cycle.

'Enrichment' systems used in this study, especially the 'static' enrichment have selected for organisms indigenous to oligotrophic environments, which have, on the whole, only been previously observed under the microscope. By the use of this simple technique, many more of these unusual bacteria can be characterised. It would appear from this study that the morphological diversity amongst bacterial genera may not be as pronounced as once thought, with the environmentally induced phenotypic variations of a few microorganisms producing a wealth of morphologically unusual bacteria.

Now that it is possible to characterise the bacteria indigenous to oligotrophic environments, and the bacterial characterisation of eutrophic waters presents few problems, one can determine a gradient of bacterial cell types in various water bodies, and consequently monitor how 'polluted' a body of water is by looking at its indigenous population, e.g. in 'clean' water prosthecate bacteria abound with well developed prosthecae (because the water is nutrient poor), whereas in 'polluted' water the prosthecate population is rapidly overgrown by bacteria which grow faster, and those prosthecate bacteria still present will lack their extensive prosthecal networks.

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